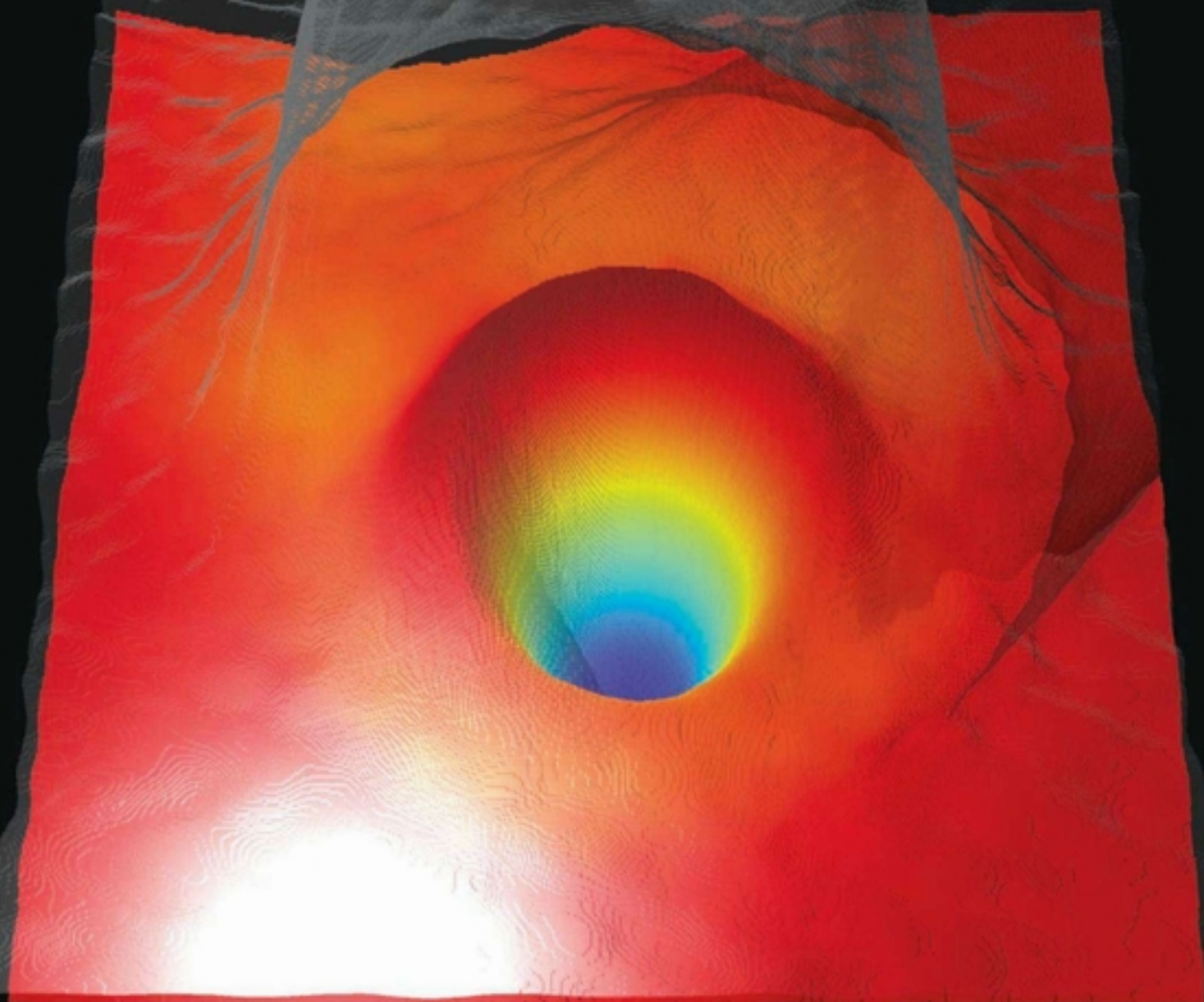


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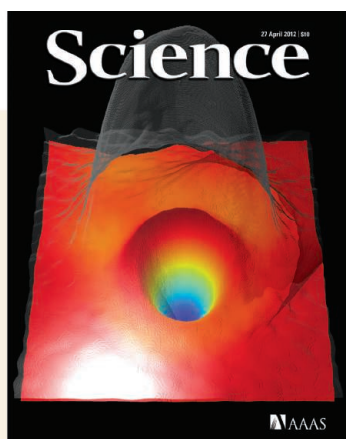
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COVER

Overlayed scanning tunneling and atomic force microscopy images of a single atom (width 1.3 nanometers): tunneling current (gray veil) and force (colored surface) between a tungsten atom and a carbon monoxide molecule. The force shows a strong angular dependence and is attractive (blue minimum) in one direction and repulsive (dark red crescent) in others. The angular dependence of single chemical bonds determines the shape of molecules and crystals. See page 444.

Image: Joachim Welker, University of Regensburg

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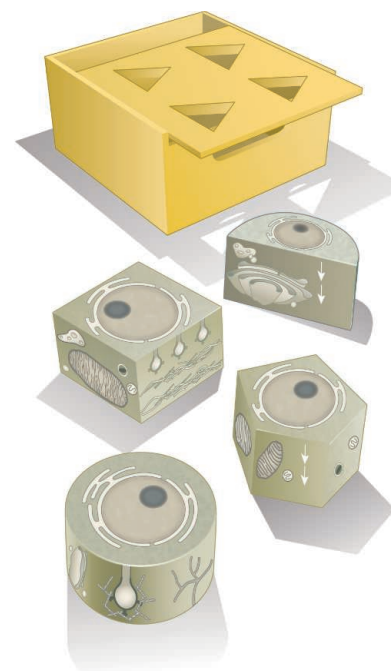
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W. Keil et al.

Full text at www.sciencemag.org/cgi/content/full/336/6080/413-d

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http://scim.ag/Comet_Wipeout

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http://scim.ag/Early_Humans

Why Creepy People Give Us the Chills

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B. Gomez-Mancilla

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Experimental Error: Thick Books and Thin Films

A. Ruben

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Another NPA Founder Speaks

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ADVANCING SCIENCE. SERVING SOCIETY



A Volcanic Valley on Mars

The formation of the Athabasca Valles, a valley network near the equator of Mars, has been debated for over a decade; both lava and water ice have been implicated. Some studies have attributed the polygonally patterned ground in this region to frost wedging in an ice-rich soil. Using images of the Athabasca region from the High Resolution Imaging Science Experiment onboard Mars Reconnaissance Orbiter, **Ryan and Christensen** (p. 449) detected numerous spirals on polygonally patterned ground. The spiral shapes are consistent with those of lava coils that form on the surface of Hawaiian lava flows, and cannot be explained by ice-related activities.

Getting Around the Block

Diblock copolymers provide a rich variety of morphologies that depend on the length of the polymer blocks, the overall fraction of each block, and their chemical dissimilarity. New synthetic methods have made it possible to make copolymers with three or more components and in a range of chemical architectures. However, this growth in design choices can offer too many variables to work with, and rational design is important, especially when trying to transform small-scale products in engineered commodities. **Bates et al.** (p. 434) review the opportunities and complexities that exist when working in this expanded playground of block copolymers.

Gut Reaction

The gut needs to keep its trillions of microbial inhabitants contained. The immune system has evolved a multifaceted approach to this problem, which includes the production of large quantities of immunoglobulin A (IgA) in the intestinal mucosa. In a process that is not well understood, plasma cells that produce IgA specific for the gut microflora are selected in Peyer's patches in the gut. **Kawamoto et al.** (p. 485) used genetically manipulated mice to show that the inhibitory co-receptor, programmed cell death-1 (PD-1), is required for the proper selection IgA-secreting cells in the gut. The effect of PD-1 deletion, however, was not intrinsic to the B cells that produce IgA. Instead, the absence of PD-1 affected the differentiation of T follicular helper cells, which provide important signals to B cells that help guide them as

they develop the capacity to produce microflora-specific IgA. Mice deficient in PD-1 exhibited alterations in the composition in their microflora, which suggests that defective selection of IgA can perturb the careful balance that exists between the immune system and resident bacteria.

Synchronize Watches

Time standards based on the energy-level transitions of atoms and ions provide the most accurate and precise methods of time keeping. Measurements made in one laboratory and in another must be done with clocks that have been synchronized and calibrated to ensure that the same measurement is being made. Such clocks, however, are not particularly mobile and are housed in national metrology labs.

Predehl et al. (p. 441; see the Perspective by **Warrington**) linked two optical clocks separated by over 900 kilometers using optical fiber to show that the clocks can be synchronized, with the clocks showing a frequency stability better than 3.7×10^{-19} . Such long-distance synchronization should allow for tests of fundamental physics, such as general relativity and quantum electrodynamics.

Working the Angles on Chemical Bonding

The forces exerted by chemical bonds depend not only on the distances between atoms but also upon the angles between them. **Welker and Giessibl** (p. 444; see the cover) probed the angular dependence of the CO molecule

adsorbed on top of a copper atom on an atomically flat Cu(111) surface using both atomic force microscopy and scanning tunneling microscopy (STM). Probe tips with three different tip-atom-symmetry environments were used. All three tips delivered similar STM images, but force probes revealed the angular dependence of the CO bond to the surface and provided data for a model of the changes in bond energy.

Space Organics

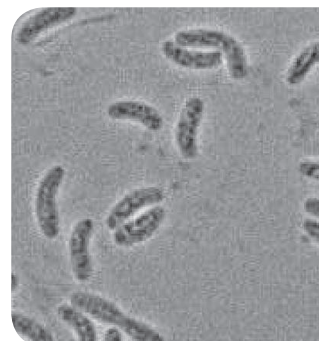
The origin of the organic compounds found in meteorites and interplanetary dust particles is a matter of debate. Laboratory experiments suggest that these organics were inherited from the interstellar medium and predate the existence of the solar system. By using particle-tracking models, **Ciesla and Sandford** (p. 452, published online 29 March; see the Perspective by **Nuth and Johnson**) explored the possibility that the organics could have been produced within the outer reaches of the protoplanetary disk from which the solar system planets originate. Grains within a protoplanetary disk follow irregular paths; such orbital histories subjected model particles to ultraviolet radiation and temperatures that have been shown in laboratory experiments to generate organic compounds.

Keep It Inside

Some cyanobacteria form solid-phase calcium carbonate precipitates as a consequence of fixing CO₂ during photosynthesis. Usually, such carbonates form extracellularly near the surface of the cells, sometimes generating structures called stromatolites.

In a biofilm growing on carbonate deposits in Lake Alchichica, Mexico, **Couradeau et al.** (p. 459; see the Perspective by **Riding**) discovered one species of cyanobacteria that also precipitates amorphous carbonate particles internally. Because the structure and chemical composition of these carbonates is distinct from those formed extracellularly, there may be cellular control over the mineralization process. These precipitates may influence physiological processes such as cell buoyancy and the sequestration of excess alkalinity generated during photosynthesis.

Continued on page 391



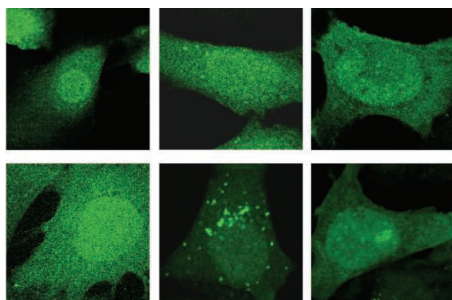
Continued from page 389

Getting Wetter Faster

Theoretical projections, based on the relationship between temperature and the amount of water vapor the atmosphere can hold, suggest that global warming should intensify the strength of the atmospheric water cycle by about twice the rate as the thermodynamics and climate models predict. **Durack et al.** (p. 455) examined 50 years of observations of sea surface salinities and conclude that the patterns of change in the salinity data are consistent with the theoretical projections, rather than with those of the models. Thus, the global water cycle should intensify by 16 to 24% for a future increase of a global average temperature of 2 to 3°C.

Farmers Displaced European Hunters

Our understanding of prehistoric demography and human evolution has been improved by analysis of ancient DNA. **Skoglund et al.** (p. 466) describe the retrieval and analysis of genomic DNA from ancient (~5000-year-old) northern European Neolithic individuals within modern Sweden. These include three hunter-gatherers from the Pitted Ware Culture horizon and one farmer ascribed to the Mid-Neolithic North-Central TRB culture. The hunter-gatherers displayed a distinct genetic signature, similar to that of extant northern Europeans, whereas the farmer's genetic signature more closely resembled southern Europeans, suggesting migration and admixture during the spread of farming.



Acetylation and Autophagy

Autophagy allows cells to digest their own components when necessary to survive stressful conditions. **Lin et al.** (p. 477) and **Yi et al.** (p. 474) describe signaling mechanisms in mammalian cells and yeast, respectively, by which autophagy is regulated by protein acetylation. In mammalian cells deprived of serum, the acetyltransferase TIP60 was activated by phosphorylation by the protein kinase GSK3 (glycogen synthase kinase 3).

TIP60's target appeared to be a protein kinase central to autophagy regulation, ULK1. This activating pathway was required for autophagy in the absence of serum, but was not needed for autophagy in cells deprived of glucose. In the yeast *Saccharomyces cerevisiae* starved of nitrogen, another acetylation mechanism was uncovered. Starvation led to activation of the histone acetyltransferase Esa1, which acetylated the protein Atg3, a key component of the autophagy machinery, thus increasing its interaction with another autophagy protein, Atg8.

Microbes: Early and Often

Epidemiological studies have suggested that the increase in the incidence of asthma and other inflammatory diseases seen in many parts of the world may be due to a reduced exposure to microbes during early childhood. **Olszak et al.** (p. 489, published online 22 March) now show that commensal microflora help to regulate the numbers and functions of natural killer T (NKT) cells in the colon and lung in mice. Germ-free mice had elevated numbers of NKT cells in these tissues and were more susceptible to chemically induced colitis and allergic asthma. Neonatal recolonization of germ-free mice with microflora prevented enhanced colitis and asthma sensitivity; however, exposure of adult mice to these conditions was not effective. Thus, early exposure to microbes has important, lasting effects on the immune system's sensitivity to inflammation.

Overthinking Religion?

Many theories of human cognition make a distinction between System I, which tends to be rapid and to rely on heuristics or rules of thumb, and System II, which tends to be more deliberative and analytic. This dual-process framework, within which both processes may operate simultaneously and competitively, has been used to explain a variety of situational influences upon decision-making.

Gervais and Norenzayan (p. 493) studied the application of a dual-process framework to religious disbelief and found that triggering analytic thinking processes through a variety of experimental manipulations resulted in a tendency for subjects to report lower levels of religious belief.

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Driven by Basic Research

ARE NATIONS INVESTING TOO LITTLE IN BASIC RESEARCH? IN THE CONTEXT OF THE GLOBAL DEBT crisis, several industrial countries have reduced their funding for this research. Even though the new understandings produced by this kind of science have long been the driving force behind an enormous number of key developments in society and the business world, many politicians seem to be entirely unaware of its importance beyond simply increasing the world's store of knowledge. Yet 400 years ago, a visionary political leader, the British statesman and philosopher Francis Bacon, emphasized that "Science discovery should be driven not just by the quest for intellectual enlightenment, but also for the relief of man's estate."

Detailed studies have shown the importance of research investments for prosperity. In the 1950s and 1960s, Robert Solow, a Nobel Prize Laureate in Economics, demonstrated that technological advancement, rather than labor and capital, is the driving force behind economic growth in industrial countries, with the introduction of new technologies accounting for about 80% of the growth in gross domestic product (GDP). More recent studies confirm this finding, particularly for the most developed countries. Philippe Aghion of Harvard University has shown that R&D investments become increasingly essential as economies approach the world's technological frontier.* In a complementary study, Hans Gersbach from ETH Zurich showed that in leading industrial nations it is basic research that serves as the essential driver of innovation for economic growth.† The scientists and problem-solvers trained in basic research laboratories frequently move from research institutions to industry, where they bring their knowledge and skills to bear on applied problems. But the new insights derived from basic research rarely lead to new products directly, more often having an indirect impact, with even striking discoveries leading to applications only after years or decades. Consider, for example, the theories of Albert Einstein, without which today's technologies such as lasers or satellite navigation systems would be unthinkable.

For business management it is risky, and given the long time periods required for practical benefit, not sufficiently profitable, for most companies to finance such curiosity-driven fundamental research. Instead, this basic scientific research must be adequately funded by governments, both to produce the skilled problem-solvers needed by the private sector and because the research results themselves deliver substantial benefits to the local economy. Analyses of patents show a strong national component of citation linkage, with inventors preferentially citing papers authored in their own country; thus, regional research investment brings local benefits. Ultimately, investments in fundamental research are paying off. It is a question of more than just conventional technologies and jobs. It is the results of this research that the world of tomorrow will build on.

Looking at these facts, it is clear that Europe and the United States (investing 2 and 2.8% of GDP, respectively) are gambling away opportunities if they do not improve their investments in R&D with an emphasis on basic research. Japan and Korea are already investing around 3.4% of GDP, and China and India are also gradually catching up. This allows these nations much more flexibility in the scope of their investments. Still more important, however, the global challenges that face us all will only be resolved with new knowledge yielded by basic research. We must explore new means of feeding nine billion people soon. We must find new ways to provide solutions to energy demand and climate change. We must discover how to maintain health in an ever-aging society. So the real question is not "Can we afford to invest in basic research?" It is "How can we afford not to?" Because, as Francis Bacon also wrote, "He that will not apply new remedies must accept new evils; for time is the greatest innovator."

— Peter Gruss

10.1126/science.1221292

*P. Aghion, *A Primer on Innovation and Growth* (Bruegel Policy Brief, 2006). †H. Gersbach, in *The New Economics of Technology Policy*, Dominique Foray, Ed. (Edward Elgar Publishing, Cheltenham, UK, 2009).



CLIMATE SCIENCE

The Way They Were

During much of the Eocene, from about 55 to 45 million years ago, vast conifer forests existed north of the Arctic Circle where no such vegetation exists today. Some paleobotanical studies have led to suggestions that the forests of eastern Asia are a good modern analog for those ancient woods, based on the similarity of tree types. However, more recent studies of environmental conditions such as mean annual precipitation and productivity in the Eocene have led others to conclude that the Arctic forests were more like those of the modern Pacific Northwest. Schubert *et al.* present reconstructions of the seasonality of paleoprecipitation, based on high-resolution intra-ring carbon isotope measurements of fossil wood, to show that the Eocene Arctic forests experienced around three times more precipitation during summer than during winter, unlike in the Pacific Northwest, where summer precipitation is much less abundant than winter precipitation. Therefore, the temperate forests of eastern Asia probably are the best modern analog for the Eocene Arctic forests. — HJS

Geology 10.1130/G32856.1 (2012).

BIOPHYSICS

Taking the Wrong Path

Most proteins fold efficiently into their functional native structures either on their own or with the help of a molecular chaperone. Despite quality-control pathways, however, misfolded proteins occur in the cell and are associated with a range of diseases such as Alzheimer's, Parkinson's, and prion disorders. Given the severe consequences of protein misfolding, there is much interest in understanding the mechanisms of misfolding for disease-associated proteins. In prion diseases, a misfolded form of the prion protein, PrP, aggregates and induces conversion of natively folded protein to the misfolded form. Yu *et al.* used single-molecule force spectroscopy to directly observe the misfolding of PrP. They observed two-state behavior with no evidence of an intermediate on the native folding pathway. Three non-native structures were accessed from the unfolded state, however. These misfolding pathways were explored more frequently than the native pathway, but the misfolded states were rarely occupied because of their instability. A mutant with higher aggregation propensity had a similar folding pathway as the native protein, but two of the misfolded states were more stable in the mutant, which suggests that these states

may be involved in aggregation. Applying this approach to other misfolded proteins will give insight into commonalities and differences between misfolding mechanisms. — VV

Proc. Natl. Acad. Sci. U.S.A. **109**, 5283 (2012).

NEUROSCIENCE

Evolving Function

Excessive expansion of a series of glutamine residues in the N terminus of the protein huntingtin (HTT) lies at the root of the neurodegenerative Huntington's disease. Although

HTT is evolutionarily conserved, the number of glutamines the gene encodes is not. For example, HTT in the slime mold *Dictyostelium discoideum* has none of the susceptible glutamines; however, the sea urchin (*Strongylocentrotus purpuratus*) variant of HTT has two. In mammals, HTT has a larger and more expandable glutamine tract and is expressed in the developing brain, where it is known to regulate neural tube formation. How HTT specifically regulates neurulation, however, is not well understood. Lo Sardo *et al.* found that mouse embryonic stem cells deficient in HTT expression were also deficient

in the intercellular adhesions needed to form neuroepithelial rosettes, hallmarks of neurulation in vitro. The authors tested the ability of N-terminal portions of HTT from other organisms to substitute for the mammalian HTT and found that HTT fragments from the evolutionarily distant *Dictyostelium* were the least effective. These results suggest a possible connection between the acquisition of a cell adhesion function during the evolution of the HTT protein and the evolution of more complex, centralized neural systems developed by the process of neurulation during embryogenesis. — PJH

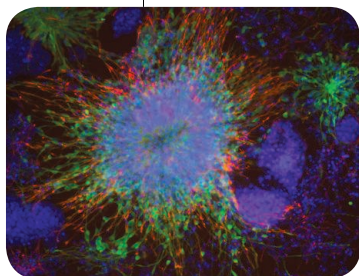
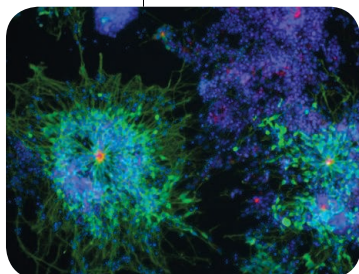
Nat. Neurosci. **15**, 10.1038/nn.3080 (2012).

MATERIALS SCIENCE

Remote Heating

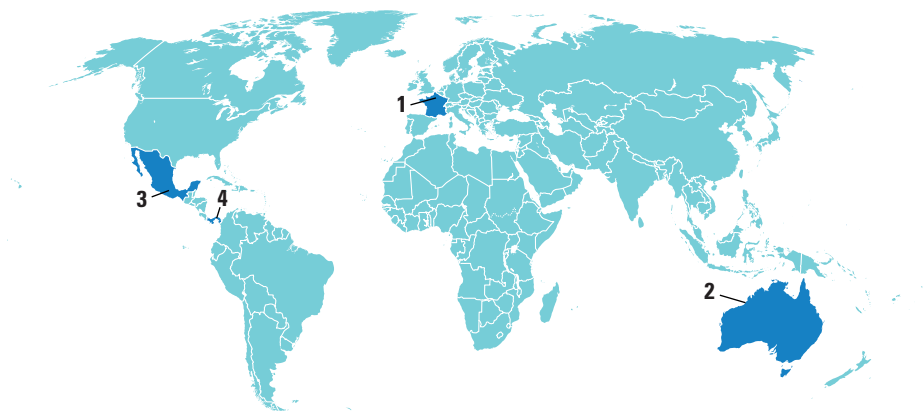
The high electronic conductivity of carbon nanotubes and graphene could be exploited for delivering power to electronic devices. However, even these good conductors can undergo resistive or Joule heating, and despite their good thermal conductivity, dissipating the heat from a device may prove problematic—interfaces with other materials appear to have high interfacial thermal resistance. Baloch *et al.* show that when current flowed through a multiwalled carbon nanotube on a substrate (a silicon nitride membrane), 84% of the heating occurred in the underlying substrate. This remote Joule heating was driven by the electrical current coupling to vibrational modes in the membrane. These conclusions are based on modeling of thermal profiles determined by following the melting of indium overlayers on these devices with transmission electron microscopy. — PDS

Nat. Nano. 10.1038/NNANO.2012.39 (2012).



CREDITS (TOP TO BOTTOM): ISTOCKPHOTO.COM; LO SARDO ET AL. NAT. NEUROSCI. 15, 10.1038/nn.3080 (2012)

AROUND THE WORLD



Paris 1

Envisat Still Offline

Things are not looking good for Europe's flagship Earth observation satellite Envisat. Ground controllers lost communication with the craft on 8 April and have been unable to reestablish contact. They have determined that the craft is

still in a stable orbit and not spinning, ruling out a collision, and that its radar antenna and solar array are both intact.

Envisat carries sensors for scanning land, sea, and atmosphere, and has been the mainstay of European environmental researchers for the past

10 years. The European Space Agency (ESA) hoped Envisat would last until ESA's next-generation Sentinel satellites are launched.

ESA now has an agreement to get radar imaging data from Canada's Radarsat. Those using Envisat's radar altimeter also have alternatives. "We at least for the time being have [ESA's] CryoSat-2 to ensure continuity of polar altimetry," says climate physicist Seymour Laxon of University College London. But for researchers studying air quality and atmospheric science "there is nothing to replace [Envisat's instruments]," says Robert Meisner of ESA's Earth observation program. The next generation of these instruments is due to fly on Sentinel 3, slated for launch in 2014.

<http://scim.ag/Envisat>



Western Australia 2

Breeding Humpbacks Get Safe Space

Every year, 20,000 or so humpback whales migrate north from Antarctica's feeding grounds to breed in the warmer waters of Western Australia. About 1000 of the whales of this "Breeding Group D" population head to Australia's Kimberley Coast, making it the largest humpback whale calving area in the Southern Hemisphere—and last week Western Australian officials announced that part of a planned marine park will be set aside to help protect the annual visitors.

The Camden Sound Marine Park will span nearly 7000 square kilometers, and will include zones closed off to commercial fishing and others to protect the region's islands, shoals, and reefs. A "special purpose zone," spanning about 25% of the park, will protect the whales, requiring vessels to stay at least 500 meters from the humpback mothers and calves.

The whales remain in the warm waters for several months, before attempting the trip back to the Antarctic summer feeding grounds.



Mexico City 3

Mexico Passes Tough Climate Change Law

Mexico's legislature passed a strong, new climate change law on 19 April. The law includes provisions to mitigate climate change and requires 35% of the country's energy to come from renewable sources by 2024. It also includes a mandate to reduce carbon dioxide emissions by 30% below current levels by 2020 and 50% below current levels by 2050. Mexico is now the second country in the world, after the United Kingdom, to have legally binding emissions goals intended to reduce the impacts of climate change.

NOTED

>Wondering how you can help prevent budget cuts to planetary science this year? Try a bake sale, says Alan Stern, of the Southwest Research Institute in Boulder, Colorado. Stern has proposed a **National Planetary Exploration Car Wash & Bake Sale** to be held 9 June as a way to raise awareness of potential cuts and how they might affect planetary research.

THEY SAID IT

"I think this a great way to show how biochemistry can daily impact their life. It also makes them a much better cook!"

—Joseph Provost, describing his science of cooking class at Minnesota State University, Moorhead, at the American Society for Biochemistry and Molecular Biology annual meeting this week

Panama City 4

'IPCC for Biodiversity' Gets Final Approval

Negotiators from 90 countries agreed this week on the final design of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES). Working under the auspices of the United Nations, IPBES will be analogous to the Intergovernmental Panel on Climate Change, creating regular assessments of the state of biodiversity around the world that will be geared toward policymakers (*Science*, 4 March 2011, p. 1139). Teams of volunteer experts will also highlight areas where more research is needed. Germany beat out several countries, including India, that are more biodiverse to host the IPBES, which will be headquartered in Bonn.

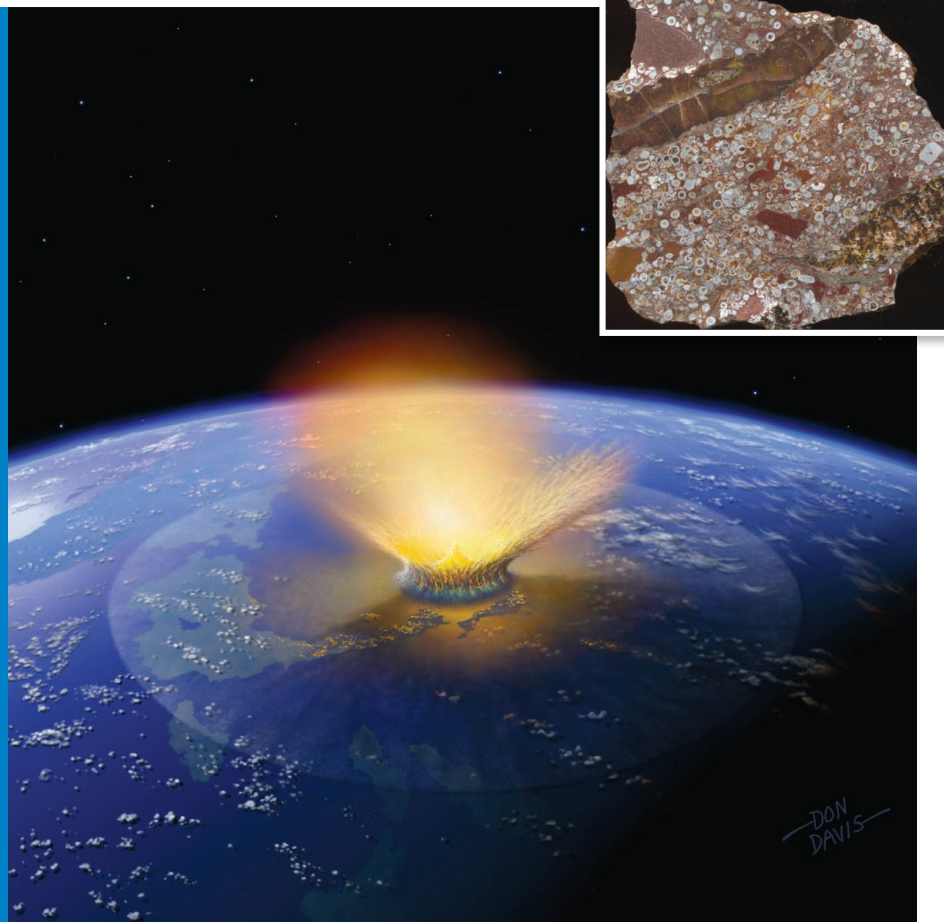
FINDINGS

Heat Held Back the Pioneers

The world is once again safe from exotic "new" physics, at least any provoked by the

Pioneer 10 and Pioneer 11 spacecraft. Something mysterious—perhaps a variation in Newton's laws—had been slightly slowing the spacecraft as they headed out of the solar system, 2 September

2011, p. 1208). Blasted away from Earth in the early 1970s at more than 51,000 kilometers per hour, both spacecraft had been slowing by 1 kilometer per hour per decade.



Heavy Hitting

The so-called Late Heavy Bombardment (LHB)—when comets and asteroids are thought to have pummeled Earth, the moon, and other inner bodies of the solar system—may have lasted longer than once thought.

One explanation for the LHB, thought to have lasted between 4.1 billion and 3.8 billion years ago, is that migration of gas giant planets sent objects in the asteroid belt zooming toward the center of the solar system. Now, in one of two new studies in *Nature* this week, researchers suggest that the LHB was prolonged by later impactors that came from a largely extinct belt of asteroids known as the E belt.

Rocks from lunar craters have provided much of the evidence for the LHB, but in the second *Nature* paper, scientists examining Earth-based evidence also found support for a longer onslaught. The flux of impactors, they report, was much higher 3.5 billion years ago than now. The new data came from spherule layers, which formed when rock vaporized by impacts condensed into molten droplets that were then preserved in the geologic record (inset).

But that minuscule deceleration bothered many spacecraft navigators, so astrophysicist Slava Turyshev of NASA's Jet Propulsion Laboratory in Pasadena, California, and colleagues calculated in unprecedented detail the net effect of the subtle forces acting on the spacecraft. In a paper to be published in *Physical Review Letters*, the group

reports that more of the heat from a Pioneer's radioisotope thermoelectric generator and from the onboard instrumentation it powered radiated in the forward direction than radiated back toward the sun. The skewed heat radiation—in effect an infinitesimal rocket—was just strong enough to apply the observed tiny braking. >>

CREDITS (TOP TO BOTTOM): (INSET) BRUCE M. SIMONSON; DON DAVIS; NASA

Random Sample

A Very Scientific Comic Book

Take one Greenlandic graphic artist and add a group of Danish archaeologists and self-confessed “cartoon nerds” from the National Museum of Denmark in Copenhagen, and what do you get? A series of action-packed graphic novels on the prehistory of Greenland, from the peopling of the

island 4500 years ago to the shamanic traditions of later Dorset culture hunters.

“We pulled together all the knowledge we could to make these subjective, fictional stories,” says Martin Appelt, an archaeologist at the National Museum of Denmark. Artist Nuka Konrad Godtfredsen discusses the storylines with the archaeologists and then bases his drawings on data such as scientific records of excavated artifacts and campsites or photographs of flint-knapping experiments. The first book in a planned series of four graphic novels appeared in 2009 in three languages: Danish, Greenlandic, and English. The second will appear in May.

Robert Park, an Arctic archaeologist at the University of Waterloo in Canada, says these graphic novels will keep aboriginal communities abreast of research conducted on their ancient sites—a form of accountability that earlier archaeologists often neglected. “In my training as an archaeologist,” says Park, “presenting archaeological information back to the Inuit communities was just not on the radar.”

Working with Godtfredsen on the illustrations has yielded scientific dividends: National Museum of Denmark archaeologist Bjarne Grønnow reexamined wooden artifacts from the 4500-year-old camp site of Qeqertasussuk and recently found that one bent and grooved strip formed the rim of an Arctic drum, pushing back the drum’s history by 3500 years.

“By having Nuka there visualizing the ideas that we have,” concludes Appelt, “it has forced us to be more specific about concepts we were once a little vague about.”

>>FINDINGS

Human Ancestors Out-Hunted Large Carnivores

When human ancestors began hunting for meat on the plains of Africa 2.5 million years ago, they apparently took more than their fair share of flesh. Within a million years, most large carnivores in the region—from saber-toothed cats to bear-sized otters—had gone extinct, according to a study presented last week at a workshop on climate change and human evolution

at Columbia University’s Lamont-Doherty Earth Observatory in Palisades, New York.

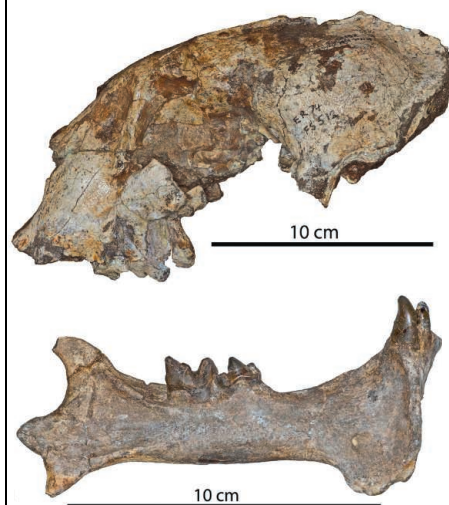
Homo erectus appeared in the fossil record 1.9 million years ago, and has been blamed for the extinction of many African mammals. To investigate when the decline began and which carnivores went extinct, vertebrate paleontologist Lars Werdelin of the Swedish Museum of Natural History in Stockholm studied the anatomy of fossils (including jaws, teeth, and snouts) of 78 species of carnivores to reconstruct their dietary breadth and analyze which types of carnivores disappeared.

BY THE NUMBERS

0.66 million km³

Total volume of groundwater in Africa, according to a new estimate based on hydrogeological maps and preexisting studies published online 19 April in *Environmental Research Letters*.

9,700 The number of people likely to die if a magnitude-7.3 earthquake occurs on a fault near Tokyo, according to a new study by the Tokyo Metropolitan Government.



Cool cats. The skull and jaw of two different species of extinct saber-toothed cats, which lived during the heyday of carnivores 3 million to 3.5 million years ago in the Turkana Basin of Kenya.

Many species of large carnivores had already gone extinct by the time *H. erectus* appeared, Werdelin found. And, he discovered, it was omnivores such as civet cats that suffered most from human scavenging by 1.5 million years ago. Only a few hyper-carnivores that ate only meat, such as lions and leopards, survived.

<http://scim.ag/largecarnivores>

Science LIVE

Join us Thursday, 3 May, at 3 p.m. EDT for a live chat with an expert on the **search for extraterrestrial intelligence**, also known as SETI. <http://scim.ag/science-live>



Ring it up. Supporters of Proposition 29 want California's smokers to pay for research on cancer and other tobacco-related illnesses.

CANCER

California Weighs Tobacco Tax Hike to Fund Research

Thanks to the unhealthy habits of some of the state's residents, California voters have an opportunity on 5 June to make their home one of the world's biggest supporters of cancer research. The ballot for the state's Republican presidential primary contains a measure, Proposition 29, that would impose an additional tax of \$1 on each pack of cigarettes sold and an equivalent tax on other tobacco products. According to a state analysis, the measure would generate about \$735 million a year in new revenue, of which \$441 million would be slated for grants and loans to support research on cancer, cardiovascular disease, and other tobacco-related illnesses. Another \$110 million would annually go toward research facilities.

"It would be unprecedented. We'd become the second largest funder of cancer research in the world, next to the [U.S.] National Cancer Institute [NCI]," says former state senator Don Perata, who authored the measure. NCI has a budget of about \$5 billion—it spent upward of \$490 million on research grants and contracts in California alone in 2011—and Proposition 29 would, at the least, put California on par with the biggest international funders, such as research charity Cancer Research UK (\$531 million annually), and ahead of Texas, which passed a 2007 bond measure that will generate \$3 billion for cancer research over 10 years.

Perata, who turns 67 next week, says he had no special interest in biomedical research until 6 or 7 years ago when he visited the Cal-

ifornia Institute for Quantitative Biosciences in San Francisco. Known as QB3, the institute is a joint venture among the University of California (UC) campuses at Berkeley, San Francisco, and Santa Cruz. QB3 Director Regis Kelly hoped to enlist Perata's help in raising money for research on new technologies that would cut health care costs. Perata says he was impressed by the pitch, but knew that term limits would force him out of office in 2008, and he wanted to create an enduring source of funding. Proposition 29 is the solution he came up with. Perata says he launched the effort to get it on the ballot with \$800,000 left over in his senate campaign fund. Then, in 2010, he was diagnosed with prostate cancer. "I'm at an age where everyone I know either knows someone with cancer or has it themselves," he says.

As Proposition 29 got off the ground, Perata consulted other scientists and organizations that support research on tobacco-related illnesses, including the American Cancer Society (ACS), the American Heart Association, and the American Lung Association. Not surprisingly, these groups all back the measure. "Given the challenges that are going on at the national level in terms of funding research and the continuing attacks on research budgets, having this money to support research here would just be tremendous," says Carolyn Bruzdinski, who directs research, clinical, and education efforts for the California division of the ACS.

"This is a very opportune time to make this investment," adds Kristiina Vuori, head of the Sanford Burnham Medical Research Institute in San Diego. Thanks to advances in DNA sequencing, personalized treatments tailored to the genetic profile of a patient's tumor are finally entering the clinic, Vuori notes. And other research areas, from preventing early stage tumors from progressing to understanding why cancer spreads, are poised to take off, she says.

It's not the research money, however, that most excites Stanton Glantz, who directs the Center for Tobacco Control Research and Education at UC San Francisco. About \$147 million of Proposition 29's annual funds would go toward smoking-cessation efforts, and Glantz says that's enough for a strong media campaign that, in combination with the price increase on tobacco products, could reduce the state's already low smoking rate even further. (Fewer than 12% of Californians smoke.) "I think that if 29 passes, we could wipe out tobacco as a public health issue within 5 years," Glantz says.

Opponents to the measure include tobacco companies and antitax groups. A coalition of groups called No on 29 says on its Web site: "We all believe cancer research is important, but California can't afford to start a new billion-dollar spending program when we have a \$10+ billion budget deficit and can't pay for critically-needed existing programs like education and health care." Critics also say the measure would create a burdensome bureaucracy rife with potential conflicts of interest.

That's because some of the people deciding where the research money goes would represent institutions that stand to benefit. Proposition 29's research funds would be administered by a nine-person committee

made up of the chancellors of UC Berkeley, San Francisco, and Santa Cruz; the heads of three of the 10 NCI Cancer Centers in the state, to be appointed by the governor; a cardiovascular physician, selected by the governor; and two patient advocates selected by the director of the state Department of Public Health. The grant-review process would closely mimic the system used by the National Institutes of Health.

"A huge research bureaucracy dominated by political appointees with no accountability," is how the arrangement is described in a radio ad from No on 29. Similar criticisms have been leveled at the California Institute of Regenerative Medicine (CIRM), funded by a \$3 billion bond measure passed by voters in 2007.

Proposition 29 supporters counter that funding decisions have to be made by people with scientific expertise and provisions written into the measure will prevent favoritism. Perata and others say some lessons from the CIRM experience were incorporated into Proposition 29, including a much leaner governing body (nine compared with 27). "The intent with Proposition 29 is to be much less bureaucratic than CIRM has been," says Vuori, who is a CIRM board member.

Critics also complain that Proposition 29 allows funds to be spent out of state, or even outside the United States. Supporters say that's necessary to enable collaborations, but they argue that the governing board would be filled with people committed to keeping the money in the state. They see Proposition 29 as a way to lure top cancer researchers and make up for a few big names lost to the Texas cancer initiative. Says Perata: "They ripped off some really good California scientists, took them to Baylor for Christ's sake!"

The most recent poll data, from a survey of registered voters conducted in late March, found that 68% of respondents supported the measure, while 29% opposed it. But that's no guarantee of victory. The last time California considered raising that tax, in 2006, tobacco companies pumped \$67 million into the opposition campaign. The measure, which would have raised the cigarette tax by \$2.60 a pack to fund health services, health insurance for children, and tobacco-use prevention programs, was narrowly defeated.

Supporters are being outspent this time, too. They've raised \$4.2 million, with ACS and the Lance Armstrong Foundation each kicking in roughly \$1.5 million. Opponents, led by a \$14.7 million contribution from Phillip Morris, have so far raised \$23.7 million to defeat the measure. In the coming weeks, the campaign seems likely to light up. **—GREG MILLER**

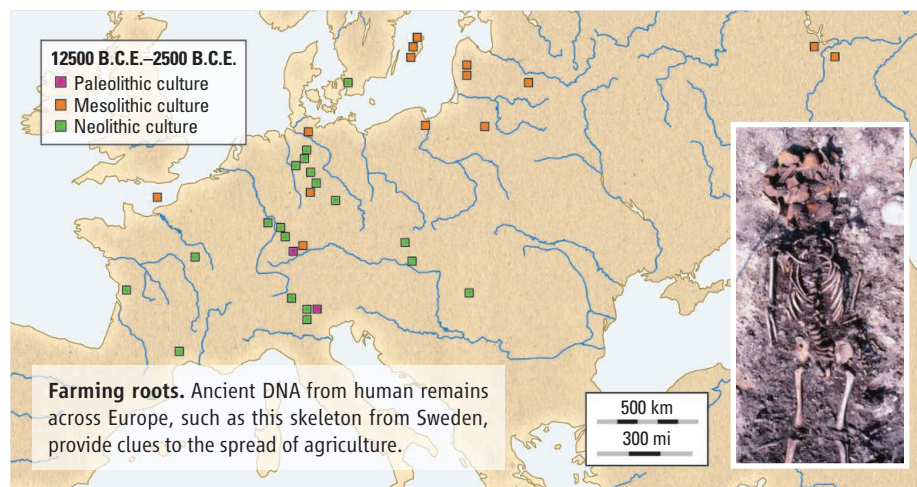
ARCHAEOLOGY

Ancient Migrants Brought Farming Way of Life to Europe

About 5000 years ago, prehistoric people living on Sweden's Gotland Island buried their dead in large collective graves according to the customs of their Pitted Ware culture, one of the last major hunter-gatherer cultures in Europe. Among the deceased were a 7-year-old child, a woman perhaps 45 years old, and a man about 25. Their graves included an assortment of artifacts such as beads made of seal teeth, shards of pottery, and a piece of pig tusk. About the same time, farmers belonging to the Funnel Beaker (TRB) culture, who lived about 400 kilometers away in what is today Gokhem parish on the mainland of southern Sweden, buried a 20-year-old woman in a megalithic tomb made of stone slabs and covered with earth.

evidence that farmers personally took the technology across Europe, and that the first farmers of chilly northern Europe came from the continent's sunny Mediterranean south.

The new findings "add a welcome piece to the big puzzle that is Europe," says Wolfgang Haak, a paleobiologist at the University of Adelaide in Australia. And because the study is based on numerous sequences of nuclear DNA, rather than segments of mitochondrial (mtDNA) or Y chromosome DNA as in most earlier work, it "really marks a turning point in the contribution of paleogenetics" to understanding both how farming spread and its legacy in the modern European gene pool, says Marie-France Deguilloux, a paleogeneticist at the University of Bordeaux in France.



Now researchers are using ancient DNA from these two sets of burials to explore the long-running question of how farming spread across Europe. Researchers agree on a few key facts: Agriculture was introduced to the continent from the Near East about 8500 years ago; it took about 3000 years for farming to reach northwest Europe; and the first farmers left detectable marks in the genomes of modern Europeans.

But many questions remain: Did farmers themselves migrate throughout Europe, or just the ideas and techniques of farming? What routes did farmers and farming take as they replaced the hunter-gatherer societies already present? Did farming advance in one solid wave, or sometimes leapfrog its way past the resident hunter-gatherers?

A study of ancient DNA from the Scandinavian burials (see p. 466) provides new

Researchers led by computational geneticist Pontus Skoglund of Uppsala University in Sweden, in collaboration with Eske Willerslev's ancient DNA group at the University of Copenhagen in Denmark, extracted nuclear DNA from the three hunter-gatherer skeletons radiocarbon-dated to between 5300 and 4400 years ago; and from the Gokhem woman, dated to 4900 years ago. Earlier isotopic analyses had shown that she was born no more than 100 kilometers south of her burial tomb and so was part of a local population.

The team sequenced a total of 249 million DNA base pairs from the four skeletons, representing about 2% to 5% of the total genome of each person. The three hunter-gatherer genomes clustered most closely with the genomes of living northern Europeans, especially Finns, while the farmer's genome was closest to those of southeastern Euro-

peans who today live in Greece and Cyprus. Skoglund and his co-workers conclude that, at least in Scandinavia, farming was brought in by genetically distinct farmers who originally hailed from the far-off south. This finding fits the overall pattern seen in several other recent mtDNA and Y chromosome studies (*Science*, 4 September 2009, p. 1189.)

As for why the prehistoric hunter-gatherers might cluster more closely with today's Finns than today's Swedes, the team points out that their ancient DNA does not closely match that of any contemporary population and may represent a gene pool that died out long ago. Some researchers have suggested that the Finns trace some of their ancestry to local hunter-gatherers rather than to farmers.

The findings support the hypothesis that "farming originated in southern Europe and spread throughout Europe into Scandinavia," says Johannes Krause, a geneticist at the University of Tübingen in Germany. He calls the new study "a huge step towards understanding how the modern genetic structure in Europe was formed." And even though the study included only a single farmer, Deguilloux says the millions of DNA base pairs the team

sequenced allowed it to compare many different ancient genes to those of modern people: "A high number of independent [genes] analyzed can substitute for a high number of analyzed subjects."

Nevertheless, at least one researcher cautions against drawing too many conclusions from such a small sample. "We are on the wrong path if we think that we could infer recent human demographic histories from genomic data on single individuals," says Joachim Burger, an anthropologist at the University of Mainz in Germany. Burger also says that identifying the Gokhem woman as a farmer is "speculative," because megalithic tombs sometimes contain much later burials by peoples who came afterward. "Nobody actually knows who is buried in megalithic graves," Burger says.

But Skoglund counters that the Gokhem woman's skeleton was directly radiocarbon-dated to 4900 years ago, making it contemporaneous with the TRB culture, and that no evidence of hunter-gatherer remains has been found in the area. And the results fit well with other recent ancient DNA work, such as a study last year showing southern origins for a large sample of early farmers in France

(<http://scim.ag/FarmingEurope>). Everything "all falls neatly into place," Haak says. He and some other researchers argue that farming took two routes into Europe from the Near East: one along the Mediterranean south and another into the Balkans and along the Danube River to central Europe. Skoglund says that although his team's study suggests a southern route for Scandinavian farmers, it is "not incompatible" with a two-route model for Europe as a whole.

The genetic distinctions between people living close together at the same time support a "leapfrog" model of the spread of farming, in which advancing farmers sometimes interbred with local hunter-gatherers and sometimes bypassed them, says Deguilloux, who discussed this model in a recent issue of *Evolutionary Anthropology*.

Nevertheless, Haak says, much research remains to be done "in every corner of the continent" before all of the pieces of the puzzle of European origins and the spread of farming can be filled in. But with recent progress in sequencing nuclear DNA, "we might arrive at a synthesis 5 or 10 years from now."

Krause agrees: "Going nuclear is certainly the way to move forward!" —MICHAEL BALTER

U.S. SCIENCE BUDGET

Spending Panels Back Boosts for NSF, NASA, NIST Programs

The National Science Foundation (NSF) and NASA got off to a strong start last week in the annual congressional budget race.

Separate spending bills in the House of Representatives and the Senate would spare both agencies from the severe cuts forecast next year for most federal agencies in the wake of last summer's budget agreement between Congress and the White House and a budget resolution approved earlier this month by the House. And while legislators aren't expected to cross the finish line for the 2013 budget until after the November elections, an early lead can't hurt.

NSF would receive a 4.2% increase under a bill marked up last week by a House spending panel. That \$299 million boost is only \$41 million short of what President Barack Obama requested for the \$7 billion agency and would fully fund its education programs. Senate appropriators fell \$100 million short of the president's request, telling NSF to protect core research activities rather than spending \$290 million more on a cluster of initiatives created in the past year by Director Subra Suresh to promote entrepreneurship, international partnerships, and out-of-the-box research ideas.

NASA's \$5 billion science mission directorate received a vote of confidence—and some guidance—from legislators. The House panel erased a cut of nearly \$200 million that Obama had proposed, leaving NASA \$5 million ahead of this year's budget, and Senate appropriators would come within \$70 million of current spending levels. Both bills would restore proposed cuts to NASA's Mars program and keep construction of the James Webb Space Telescope on schedule. The House bill emphasizes the importance of a Mars sample-return mission, which NASA says it can no longer afford. In addition, the Senate bill would transfer authority—and \$1.6 billion—for building and managing four weather satellite programs from the National Oceanic and Atmospheric Administration to NASA.

The House panel would also give the National Institute of Standards and Technology (NIST) \$80 million of the president's requested increase of \$105 million, which would be a jump of 14%. (The Senate would provide NIST with a \$75 million boost.) The endorsement of research by the Republican-led House at those three agencies is especially impressive given that it adopted a government-wide spending blueprint that provides

On track. Webb telescope funding advances.

\$19 billion less for discretionary programs than does the bipartisan Budget Control Act enacted last August. That meant the House panel had \$700 million less to spend than its Senate counterpart.

The Department of Energy's (DOE's) research programs didn't fare as well in a third House spending bill marked up last week. The bill would give DOE's Office of Science \$50 million less than the \$4.87 billion it now receives and \$168 million less than the president has requested. The budget of the Advanced Research Projects Agency—Energy would also shrink, from its current \$275 million to \$200 million. The White House had requested \$350 million for the 3-year-old agency, whose mission is to make major advances on a slew of energy technologies. The House panel also nixed Secretary of Energy Steven Chu's request for a sixth energy research center, or "hub," but it put money back into the domestic fusion program that the Administration had proposed to cut.

—JEFFREY MERVIS





Healthier future. Children like these in rural Sichuan may benefit from China's nutrition initiative.

PUBLIC HEALTH

Despite Gains, Malnutrition Among China's Rural Poor Sparks Concern

BEIJING—From SARS to bird flu to a lethal new bunyavirus, China is a crucible for deadly pathogens. But to Wang Yu, director of the Chinese Center for Disease Control and Prevention here, no microbial scourge now in circulation poses as stark a threat to society as malnutrition.

One of the most visible manifestations of malnutrition is stunting, defined as being two or more standard deviations below World Health Organization (WHO) standards for median height by age. Stunting rates in China have declined dramatically, from 33.1% in 1990 to 9.9% in 2010. However, recent trends trouble health officials here. After the global economic crisis in 2008, stunting rates rose in poor areas of rural China. And despite the tremendous gains in the past 2 decades, nearly 6.5 million children under age 5 in China are stunted.

Stunting in early childhood has grave and lasting consequences. "The data linking stunting to poor cognition, low adult wages, and lost productivity are very solid," says Mercedes de Onis, coordinator of WHO's Growth Assessment and Surveillance Unit in Geneva, Switzerland. China draws much of its labor force from the countryside. "When the new generation grows up, it will be too small and too weak to support society," Wang argues. "China's future depends on the country's ability to provide adequate nutrition."

In an initiative now underway, Chinese CDC is seeking to improve nutrition for

infants and young children, especially in the countryside. "We will systematically collect data to make people realize the severity of the challenge," Wang says. Toward that end, Chinese CDC this year plans to double the 100-strong scientific staff of its Institute of Nutrition and Health. The agency also intends to expand a pilot project carried out in Gansu Province several years ago, in which infants whose diets were supplemented with whole-fat soybean flour fortified with micronutrients scored higher on intelligence tests and had better motor skills than infants given rice flour or no supplements. "This is the first time the child nutrition issue is on the government's agenda," says Chinese CDC nutritionist Chen Chunming, former president of the Chinese Academy of Preventive Medicine.

Experts warn that food security could erode, threatening recent gains in China and the developing world. Across Asia, WHO estimates, the number of stunted children under age 5 fell from 190 million in 1990 to 100 million in 2010; prevalence receded from 49% to 28%. The prevalence of underweight young children also declined, from 33.8% in 1990 to 19.5% in 2010. China has logged similar progress since establishing a childhood nutrition surveillance system in the early 1990s. In synchrony with China's torrid economic development, stunting prevalence in young children in poor rural areas fell from 36% in 2000 to 18.9% in 2008, Chen and CDC colleagues reported in the July-August

2011 issue of *Biomedical and Environmental Sciences*. Until 2008, Chen says, "we saw a really dramatic change."

She and others expected China to protect those gains after the 2008 economic crisis, as massive stimulus spending kept the country's economy growing at a healthy clip and the government implemented measures to keep farms productive. On the whole, China fared well: Chinese CDC estimates that nationwide, the number of stunted children under 5 years old receded from 8 million in 2005 to 6.5 million in 2010. Parsing the data further, however, Chen's team found that in poor rural areas, stunting prevalence under age 5 rose from 18.9% in 2008 to 20.3% in 2010. In infants under 12 months in those areas, stunting nearly doubled from 2008 to 2009.

Malnutrition's pernicious effects are apparent to anyone who ventures into China's impoverished countryside, where "many people have very small bodies," Wang says. "Children who are 12 or 13 years old look like 8- or 9-year-olds," he says. "Body development is lagging, and most children never catch up."

Wang is not alone in hoping to steer the nation onto a more robust trajectory. Last autumn, the education ministry announced a \$2.5 billion, 5-year program to improve school meals for 26 million kindergarten through 12th grade students in 680 poor counties in China. That program will help, Wang says, but by the time children are in school, he cautions, "it's too late" to head off stunting-related deficits.

To eke out further gains, China may look to Brazil, which slashed stunting rates in its impoverished northeast from 34% in 1986 to 6% in 2006. "The Brazilian experience shows that the scourge of chronic malnutrition can be rapidly reduced if income among the poor rises and simultaneously there is increased access to schools, clean water, sanitation and basic health care," de Onis and colleagues reported in the January issue of *Public Health Nutrition*.

China is not as resource-rich as Brazil, Wang says: "We don't have enough farmland and in particular enough water." China may have to ratchet up imports of staples while better utilizing its own scarce resources. Bearing that in mind, Wang is reaching out to other ministries. "We have to integrate our nation's expertise in nutrition, agriculture, and economics," he says.

For the sake of the next generation, Chen says, China cannot delay: "It's crucial to act immediately."

—RICHARD STONE

FRANCE

Sarkozy's Foe Would Soften Reforms, Overturn Stem Cell Law

PARIS—With a fiscal crisis looming, unemployment approaching 10%, and a fresh national trauma from two shootings that left seven people dead, it's not surprising that research and higher education are playing almost no role in France's presidential election. The topic is entirely absent in President Nicolas Sarkozy's election program, and apart from a one-liner about the research tax credit (CIR) for the private sector, Sarkozy has not discussed research since he announced his reelection bid in February. "These are not vote-getting issues," one campaign adviser told *Science*.

Socialist François Hollande, who won the first round of France's presidential elections on Sunday and will now face Sarkozy in a 6 May runoff, hasn't made science a top campaign issue, either. But from a speech he gave on 5 March and interviews with advisers, it's clear that should he win—polls give him a clear advantage—France's first Socialist president since 1995 would soften the impact of some of the higher education and research reforms started by Sarkozy and his predecessor Jacques Chirac, and would try to repeal France's ban on embryonic stem cell research.

The scientific community remains divided on Sarkozy's 5-year reign. Some say his attempts to make the country's universities and national research institutions more competitive were needed, but Sarkozy also enraged French scientists with a 2009 speech in which he suggested they were lazy and denounced the research system as "infantilizing and paralyzing."

Sarkozy's research and higher education policy has been marked by two major initiatives: a law granting universities autonomy from government interference, passed with remarkably little opposition in his first year as president, and a massive funding stimulus, the Big Loan, later renamed Investments for the Future. Devised in 2009 when the economic crisis started to bite, the scheme allocates €21.9 billion for research projects, new equipment, campus facelifts, and the Excellence Initiative, aimed at creating up to 10 world-class research and higher education clusters. Eight have made the cut (*Science*, 10 February, p. 644).

If Sarkozy pulls off an upset—and his party, the Union for a Popular Movement, retains its majority in the parliamentary elections on 10 and 17 June—he will likely continue his focus on promoting excellence and project-based funding. The university auton-

omy law "means that the basics of needed reform are in place," says Sarkozy's adviser, who asked not to be identified. Sarkozy would try to keep the overall research and higher education budget stable, the adviser says, but new Investments for the Future awards are out of the question.

Researchers' trade unions and *Sauvons la Recherche*, a left-leaning researchers' movement, have put pressure on Hollande to change course radically. They say the university autonomy law and Investments for the Future are creating disparities between universities, as does the National Research Agency (ANR), France's first government granting agency, established under Chirac. The stimu-

onic stem cells. The French biomedical research agency can allow dispensations for work that could lead to medical breakthroughs, but the agency is under pressure from opponents of stem cell research to deny such dispensations, says Marc Peschanski, director of the Institute for Stem Cell Therapy and Exploration of Monogenic Diseases in Evry. Peschanski recently had two applications turned down, himself. Repealing the ban would remove the ambiguity and change "the way this research is regarded in France and abroad," Peschanski says.

Either politician will likely have to make deep cuts in France's budget, and Hollande hasn't promised researchers new money.



Left turn. François Hollande (*left*), who is ahead in the polls, has promised to help the losers in Nicolas Sarkozy's Investments for the Future competition.

lus program has also masked lagging lab budgets, they say, and most of its money is parked in accounts from which only the modest interest can be spent.

In his speech in March, Hollande made clear that he won't reverse the process of university autonomy and will honor commitments already made in the stimulus package. "The state must keep its word," says biochemist Jean-Yves Le Déaut, a member of the National Assembly and Hollande's main research adviser. But Hollande did promise to help the losers in the stimulus program in order to redress regional and other disparities. He would also water down ANR's role by giving the state responsibility for defining strategic priorities, and he would shift funding from project to basic research, which he says has been the "big sacrifice of recent years."

Hollande also publicly promised he would ask Parliament to overturn a law, revised in 2011, that largely bans research on embry-

"First, we would carry out an audit on the present government's higher education and research commitments, as we estimate there will be a shortfall of at least €2.8 billion by next year, which is a time bomb waiting to explode," Le Déaut says. "Then we would hold a national congress in December or January to decide on the content of a framework law to replace the [university autonomy law] and on other measures."

Those pledges have mostly pacified Hollande's left-wing critics, but some want concrete action as soon as possible. The scientific researcher trade organization SNCS-FSU has asked Hollande to urgently channel unspent ANR and CIR funds into labs if he wins. Labs' operating budgets have plunged by an average of 20% in both 2011 and 2012, says biochemist and SNCS-FSU general secretary Patrick Monfort. "Most of them have little cash left for running costs."

—BARBARA CASASSUS

Barbara Casassus is a writer in Paris.

PHYSICS

Textbook Electrodynamics May Contradict Relativity

A basic equation of electricity and magnetism is wrong, one scientist claims. The classic formula for the force exerted by electric and magnetic fields—the so-called Lorentz force—clashes with Einstein's special theory of relativity, says Masud Mansuripur, an electrical engineer at the University of Arizona in Tucson. Others doubt the claim but have not found a flaw in the simple argument that challenges century-old textbook physics.

"If it's true, it's astonishing," says Stephen Barnett, a theorist at the University of Strathclyde in Glasgow, U.K. "I suspect there is something subtle going on here" that doesn't contradict relativity. But Rodney Loudon, a theorist retired from the University of Essex in the United Kingdom, says,

From the particle's perspective, things look very different. In that "frame of reference," the particle stands still while the wire moves. The wire still exudes a magnetic field, but because the particle has no velocity it feels no magnetic force. Yet relativity demands that if an observer in one frame of reference sees a force, an observer in another frame should see an equal force.

A contradiction? Not quite, thanks to special relativity's weird prediction that observers moving at different speeds perceive lengths differently. Those lengths include the distances between the positively charged ions that form the wire and the negatively charged electrons that flow to produce the current. In the lab frame, the wire is stationary, and the ions and electrons are equally

magnet and the charge glide past together (figure, bottom right). The magnet appears to be electrically polarized, with a positive charge on one side and a negative charge on the other. That's because in classical electrodynamics, magnetism originates from hypothetical loops of "bound" current within a material. So the magnet is equivalent to a ring of wire carrying current in a circle. As the ring coasts by the observer, contraction effects will redistribute the charges in it just as they did in the current-carrying wire in the first example. On the side of the loop in which current flows in the same direction as the loop's motion, a positive charge appears. On the other side, a negative charge appears.

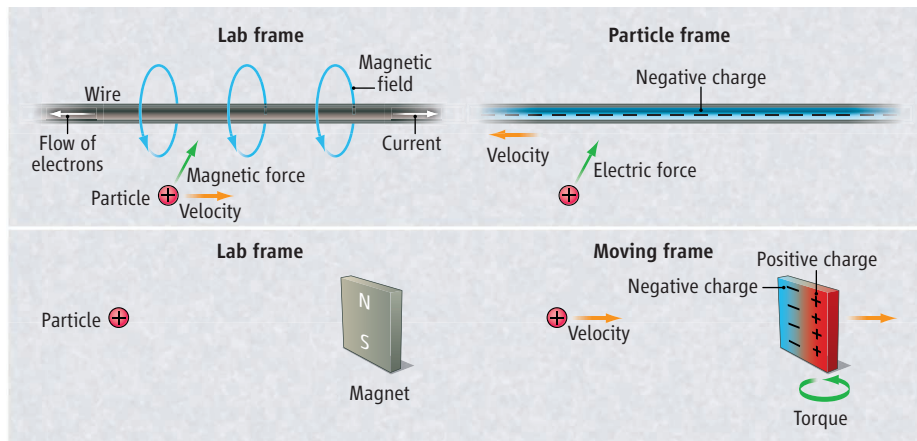
The charged particle interacts with these charges, pulling on one side of the magnet and pushing on the other to create a twisting "torque." The moving charge also produces a magnetic field, but that field does not counteract the twisting. So there's a net torque not seen in the lab frame, Mansuripur calculates. That violates relativity.

There is a way out, Mansuripur says: No torque appears in either frame if he uses a more complicated formula for forces in polarized and magnetized materials that Einstein and Jakob Laub proposed in 1908 but Einstein later repudiated. Some theorists say that's fine with them. "Einstein-Laub is correct—shock and horror!" says Daniel James of the University of Toronto in Canada.

But there's a deeper issue. In classical electrodynamics, physicists assume that magnetization and polarization originate in microscopic bound currents and charges within materials. If that's true and the Lorentz formula is correct on the microscopic level, then they must apply it to macroscopic materials, too, and run afoul of relativity, Mansuripur argues. So, he says, physicists must scrap bound charges and currents and consider polarization and magnetization fundamental entities themselves.

Them's fighting words to some. "The microscopic picture of electrodynamics is clear," James says, "and if the macroscopic picture of electrodynamics doesn't follow from that, I'd be surprised." Somehow, the Einstein-Laub equation for macroscopic materials must follow from the Lorentz force applied on the microscopic level, he says. Barnett says "there's going to be a heated debate about this result." Undoubtedly.

—ADRIAN CHO



Hit and miss. Simple examples show how the Lorentz force jibes (top) and clashes with relativity.

"As far as I can tell, [the analysis] is right."

The Lorentz force formula describes how electric and magnetic fields push around a charged particle. The electric field pushes the particle with a force proportional to the particle's charge and the field's strength. (A negatively charged particle feels a pull.) The magnetic field shoves the particle sideways in a direction perpendicular to both the field and the particle's velocity. That magnetic force is proportional to the charge, the velocity, and the field strength.

Ironically, physicists invoke the Lorentz force in the textbook example of how electrodynamics and relativity mesh. A positively charged particle moves parallel to a wire carrying current in the same direction (see figure, top left). The current produces a magnetic field that wraps around the wire. As the particle crosses the field, it feels a magnetic force pulling it toward the wire.

spaced. In the particle's frame, however, the wire moves and its ions appear more closely spaced than they are in the lab frame. But the oncoming electrons move faster still and appear even closer together. The wire thus has a net negative charge (see figure, top right). That charge draws the particle with *electric* force equal to the magnetic force seen in the lab frame. Paradox averted.

Now, an equally simple example shows how the Lorentz force trips up when applied to magnetic particles, Mansuripur argues in a paper in press at *Physical Review Letters*. A charged particle and a tiny magnet sit apart in the lab frame (see figure, bottom left). The uncharged magnet cannot feel the charged particle's electric field, and the motionless particle cannot feel the magnet's magnetic field. So no forces are at work.

Now consider how things appear to an observer in a "moving frame" in which the

GLOBAL WARMING

The Greenhouse Is Making the Water-Poor Even Poorer

How bad will global warming get? The question has long been cast in terms of how hot the world will get. But perhaps more important to the planet's inhabitants will be how much rising greenhouse gases crank up the water cycle. Theory and models predict that a strengthening greenhouse will increase precipitation where it is already relatively high—tropical rain forests, for example—and decrease it where it is already low, as in the subtropics.

A new study of the ocean's changing salinity on page 455 confirms that this “rich get richer” mechanism of water-cycle amplification has been operating for the past half-century. “It’s a pretty striking result,” says oceanographer Raymond Schmitt of Woods Hole Oceanographic Institution in Massachusetts. The result also suggests that the water cycle is intensifying quickly under global warming—twice as fast as climate models have been predicting.

Several studies of how fast water has been evaporating and falling back as rain on a global scale had suggested an acceleration of the water cycle, but they had their limitations. Using rain gauges on land was tricky given that heavy rains can be sporadic and gauge networks have been sparse. Satellites can survey the globe, but they have been at it for only a couple of decades.

So oceanographer Paul Durack of Lawrence Livermore National Laboratory in California and colleagues from the Commonwealth Scientific and Industrial Research Organisation's Marine and Atmospheric Research division in Australia looked to the ocean. After all, “that’s where the water is,” as Schmitt puts it. The oceans cover 71% of the globe, hold 97% of its water, and receive 80% of its precipitation. And gauging the oceans’ changing salinity provides a way of tracking water as it cycles between atmosphere and ocean. If more rain falls over an ocean than water evaporates from it, surface salinity drops proportionately. If evaporation outpaces rain, salinity rises.

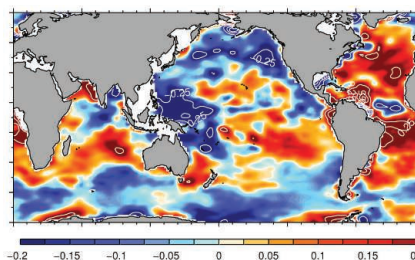
Oceanographers were not surveying global salinity intensively until 1999, when they started releasing instrumented sub-

face floats under the Argo program. Argo floats now number about 3500. But because the ocean smooths out rainfall's patchiness, even pre-Argo measurements reflect changes in the global water cycle more accurately than the denser data available over land. Durack and colleagues used the Argo observations to help correct for shortcomings in the salinity measurements made from research vessels between 1950 and 2000, such as oceanographers’ understandable reluctance to brave the



Saltier or less so.

Ship-borne instruments (top) showed that places already saltier than average got saltier (reds) and those less salty got even more so (blues).



stormy wintertime high latitudes.

When Durack and colleagues analyzed the 1.7 million salinity measurements made worldwide in the second half of the 20th century, they found that the water-rich had indeed been getting richer and vice versa (see figure). High-latitude and equatorial parts of the oceans, where greater precipitation keeps surface waters less salty than average, became even less salty; the central regions of ocean basins, where evaporation dominates and turns water saltier, became even saltier. Because 80% of the water cycle operates over oceans and much of the water falling as rain over land comes from the ocean, the water cycle over land no doubt is

behaving the same way.

After comparing the magnitude and geographical pattern of salinity change in models and in the real world, Durack and colleagues concluded that the water cycle had sped up roughly 4% while the surface warmed 0.5°C. That 8% increase per degree of warming is about the rate of acceleration expected on the basis of how much more water vapor warmer air can hold. The more moisture air can hold, the faster water can make the circuit of the water cycle, like building a bigger pipe in a plumbing system. But the warming seems to have intensified the real world's water cycle twice as much as the typical global climate model does. At least some of the models, it seems, do not properly simulate the water cycle's response to warming.

The new analysis “has provided decisive evidence for a surprisingly rapid acceleration of the global water cycle,” Schmitt says. “This is the first time we’re seeing this for the hydrological cycle,” adds oceanographer Sydney Levitus of the U.S. National Oceanic and Atmospheric Administration (NOAA) in Silver Spring, Maryland. The pattern and rate of the change “represents yet another ‘fingerprint’ of global change.”

So wet places have been getting wetter while dry places got drier. But worse is yet to come. If the world warms 2°C to 3°C by the end of the century, as currently projected, and the past is any guide to the future, the water cycle will accelerate 16% to 24%, Durack and colleagues point out. “That’s a pretty amazing projection,” says ocean modeler Stephen Griffies of NOAA's Geophysical Fluid Dynamics Laboratory in Princeton, New Jersey, “and it just might be close to reality.”

Such a revved-up water cycle would have “a lot of implications for how extreme events would change in a warming climate,” says meteorologist Brian Soden of the University of Miami in Florida. Water cycling from the surface to the atmosphere carries heat energy that can ultimately fuel violent storms, from tornadoes to tropical cyclones. The faster water cycles, the more abundant and more violent those storms might be. And wet places getting wetter can lead to more severe and more frequent flooding. Dry places getting drier would mean longer and more intense droughts. The Argo flotilla should have an interesting tale to tell in the years to come.

—RICHARD A. KERR



Europe's Embarrassing Problem

Measles, an easily preventable disease, has made a comeback in one of the richest parts of the world. Public health experts are unsure how to fight back

IN APRIL 2009, A YOUNG MAN TRAVELED from Germany to the Razgrad district in northeastern Bulgaria carrying more than his suitcase and the money he had made as a construction worker in Hamburg: He was infected with the measles virus. Upon arrival, he touched off a wave of cases that spread from the northeast of the country to the southwest, sickening more than 24,000 people and killing 24. From Bulgaria, the strain, dubbed D4-Hamburg, was carried back to Germany, and also to Turkey, Greece, Macedonia, and other European countries.

The chain of events, recently pieced together by European scientists using genetic and epidemiologic data, is typical for Europe's struggle against measles, a disease that is easy to prevent and that the continent once pledged to eliminate by 2010. While every country in the Americas, including its poorest, wiped measles off the map in 2002, Europe has been unable to do so—on the contrary. Cases have quadrupled since 2009; France alone had more than 15,000 last year.

The reemergence has become a threat to other countries. In 2011, the United States

had 222 cases, the Centers for Disease Control and Prevention reported last week—very few compared with Europe, but it was the highest number since 1996, and most importations come from Europe. “I feel very ashamed,” says Marc Sprenger, the director of the European Centre for Disease Prevention and Control (ECDC) in Stockholm. Luckily, high vaccination rates in the Americas prevent most imported infections from spreading.

Measles' stubborn persistence in Europe would also be a stumbling block in any plan to eradicate the disease globally. “The problem in Europe is actually more serious” than elsewhere, says Diane Griffin, an immunologist and long-time measles expert at Johns Hopkins University in Baltimore, Maryland. “This is what will keep measles from being eradicated, I think.”

Faced with the grim numbers and amid fears that two huge sporting events this sum-

mer could help the disease spread further, public health experts are campaigning hard to raise awareness of the dangers of measles. ECDC has made it one of its 2012 priorities, and several countries are organizing catch-up campaigns to ensure that people are getting the second vaccine dose necessary for full protection.

Moving the goalposts

One factor hampering control is the perception that measles is a harmless childhood disease. In fact, it's one of the deadliest infections in human history: Measles is estimated to have killed more than 2 million children each year before vaccines became available in 1963. Infection leads to a prolonged weakening of the immune system, and most measles deaths in the world are due to secondary bacterial or viral infections that are rarely lethal in rich countries. That's why India and sub-Saharan Africa have the highest measles mortality (see sidebar). But measles can also kill by itself; about one in 3000 cases in Europe is still fatal.

There is an effective and inexpensive vaccine, however. Two doses, the first given between 11 and 14 months of age and the second between 15 and 23 months, protect more than 98% of vaccinated children at less than \$1 per child. Most countries in Europe use a

Online

sciencemag.org

Podcast interview
(http://scim.ag/pod_6080) with author
Kai Kupferschmidt.

Protected. Emma, 15 months old, gets vaccinated against measles and three other diseases in Berlin.

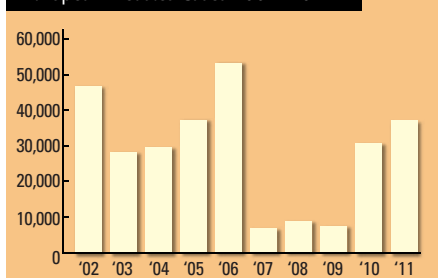
combined vaccine against measles, mumps, and rubella (MMR).

Because the virus has no other host than humans, measles could in theory be eradicated through mass deployment of the vaccine, a solution that experts have talked—and dreamed—about for decades. The success in the Americas has shown that it's possible, and in 2002, WHO's European Region—which includes Russia, Ukraine, and Turkey—set a deadline of 2010 for elimination, the regional equivalent of eradication.

It didn't even come close. Although cases fell initially, they started to climb again in 2009. In September 2010, WHO moved the goalposts by agreeing on a new 2015 deadline. But that, too, now seems close to impossible. There were about 37,000 reported measles cases in the European Region in 2011. (More than 30,000 were in the European Union, the richest part of the region.)

And public health experts are worried that 2012 might see another spike because of the European Football Championship in June. Up to a million spectators are expected to converge on Poland and Ukraine, the cup's hosts. Ukraine has already seen more than 7000 cases this year, and the peak transmission season is just starting. ECDC is urging football fans to make sure they are vaccinated. The Olympic Games in London, a month later, could become another viral hub.

European Measles Cases 2002–2011



A rash of new cases. Measles has been on the rise in WHO's European Region since 2007.

Making it mandatory

Europe's failure has multiple and complex causes. To protect an entire population against measles, at least 95% of its members needs to be vaccinated, which has proven very difficult. Many parents underestimate the risk of the disease and overestimate the vaccine's side effects. Allegations by British gastroenterologist Andrew Wakefield that the MMR vaccine can lead to autism were particularly damaging; in response, vaccination rates plummeted in the United Kingdom and Ireland. The connection was never replicated, and Wakefield's work was later shown to be fraudulent. But the scare resulted in a generation of children that is less protected.

Anthroposophic communities and certain Protestant churches are opposed to vaccinations on philosophical or religious grounds. Minorities and marginalized groups, such as the Roma, can be hard to reach, as well. And

there are more practical reasons for why children don't get vaccinated, says Ole Wichmann of the Robert Koch Institute, the German center for disease prevention and control: "One of the most common is that parents simply forgot it or did not have the time."

Similar obstacles occur in the Americas, however, and experts have difficulty explaining the huge gap in success. A key factor may be that in the United States and many countries in Latin America, children must be vaccinated before they enter school, Griffin says. "There is no reason to think we wouldn't have the same kinds of problems in the Americas, if vaccination wasn't mandatory," she says. But Sprenger and Wichmann say that would run into major legal and ethical problems in Europe and would not be accepted. But it would help if health officials were allowed to go into schools to offer vaccination, Wichmann says.

Scientists are looking for other solutions, as well. "We need to use social media more," Sprenger says. "We need to look for new approaches." ECDC is bringing together professionals in public relations, marketing, and communications at a "freethinkers meeting" this month to brainstorm about new ways to promote measles vaccination.

Perhaps simply making life easier for parents would help, says Robb Butler of WHO's European Regional Office: "We lead these fast and furious lives now, but immunization campaigns haven't changed." He suggests offering parents an opportunity to get their children vaccinated after working hours or giving workers a few hours off for the shots. "It's all posters, brochures, and stickers," Butler says. "But a sticker won't change a thing if you do not have the time."

ECDC is also trying to get doctors more involved. "Health professionals have a huge responsibility. There is no room for complacency," Sprenger says. Doctors and nurses also need to be vaccinated themselves, Wichmann says; not all are. "If they are not, they shouldn't be allowed to work in intensive care units or oncological wards where there are vulnerable patients," he says.

The tragedy is that countries of the First World appear to have forgotten how important vaccination is, says Seth Berkley, director of the GAVI Alliance. His wife, a physician who ran the intensive care unit of a major teaching hospital in New York, has never seen a case of measles, Berkley says. "I have been in a refugee camp and watched measles come through, and every day I saw the little graves of all the babies who were buried. You don't forget that."

—KAI KUPFERSCHMIDT

Kai Kupferschmidt is a science writer in Berlin.

After a Successful Decade, Global Fight Appears Stalled

It's not just Europe that has trouble getting measles under control. A new report from the World Health Organization (WHO), the U.S. Centers for Disease Control and Prevention (CDC), and Pennsylvania State University estimates that global measles deaths declined dramatically between 2000 and 2007, thanks in part to a new partnership called the Measles Initiative. But since 2007, the numbers have essentially remained flat. The report, published in *The Lancet* this week, estimates that 139,000 children died of measles in 2010—an impressive 74% reduction since 2000, but short of WHO's goal of 90%.

The global recession is largely to blame, says Stephen Cochi of CDC, who was not involved in the study. Funding for the Measles Initiative, in which CDC is a partner, dropped by 75%, Cochi says; as a result, campaigns were delayed, target groups were narrowed, and corners were cut.

To arrive at the estimates, the scientists used a new method that integrates data about vaccine coverage and efficacy with actual surveillance numbers, says co-author Peter Strebel, a measles expert at WHO. "These are the best estimates we have ever had," Strebel says. Africa saw the greatest reduction in mortality—from 337,000 in 2000 to 50,000 in 2010. Deaths dropped from an estimated 88,000 to 66,000 in India, which now accounts for almost half of the global mortality.

The paper is another reality check—along with Europe's troubles (see main text)—for those hoping to eradicate measles. WHO has not set a deadline for eradication, but five of the six WHO regions—the entire world minus Southeast Asia—have pledged to eliminate the disease by 2020.

Still, measles researchers feel confident that mortality will drop further eventually. Funding for the Measles Initiative has picked up, Cochi says. India recently adopted a two-dose vaccination strategy that provides optimal protection, and numbers there are bound to go down. "They have made great progress in 2011 and 2012," Strebel says. "That is not yet captured in the data."

—K.K.

EVOLUTION OF LANGUAGE

Experiments Probe Language's Origins and Development

In a new twist for an old field, language researchers are heading to the laboratory to test hypotheses

KYOTO AND TOKYO—Playing slide whistles. Learning fictitious “alien” languages. Making Stone Age tools. Those all sound more like hobbies than scientific pursuits. But such activities are at the heart of recent efforts to understand the emergence and evolution of language. And the trend shows how much the field is changing.

Theorizing once dominated work on the origins of language. More recently, researchers have gone into the field to study how songbirds learn to sing and into nurseries to observe the vocalizations and gestures of children for hints of how language may have emerged. Now researchers are testing their hypotheses under experimental conditions. “Five or 6 years ago, it seemed an odd idea that we could do experiments in language evolution, but that has changed,” says Simon Kirby, an evolutionary linguist at the University of Edinburgh in the United Kingdom.

The experiments, observations, and even some theorizing were on the agenda at the Evolang9 conference in Kyoto and a follow-up forum in Tokyo last month.* By design, these meetings bring diverse views together to unravel questions not likely to be answered by work within one discipline (*Science*, 21 May 2010, p. 969).

Cognitive tools

Evo-devo, or evolutionary developmental, theorists as far back as Charles Darwin in *The Descent of Man* have speculated that there may be a connection between language and stone toolmaking (*Science*, 6 February 2009, p. 709). “There is a rich line of people who tried to look at the archaeological record of the making of stone tools and the evolution of language,” says Michael Arbib, a neuroscientist at the University of Southern California in

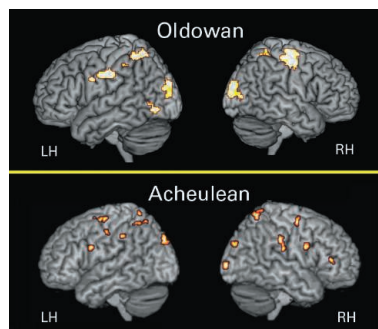
Los Angeles. “You look at the archaeological relics,” he says, and “try to infer the behavior involved” in making them.

The inferences start with the earliest known examples of human technology, Oldowan cutting tools. Dating back 2.6 million years, they are simple stone flakes with sharp edges knapped off crude “cores” using “hammerstones.” Such tools gradually became more refined, achieving a high level of sophistication about



Knapping know-how.

Archaeologist Bruce Bradley reproduces Late Acheulean stone axes similar to one from 500,000 years ago (top right). Brain imaging (right) shows that stone toolmaking activates areas also involved in language.



700,000 years ago with Late Acheulean handaxes. High-tech by comparison, these were deliberately crafted into oval or tear-drop shapes in multistep manufacturing processes that required planning and significant skill. One hypothesis is that the cognitive capabilities that supported toolmaking gave the toolmakers language-ready brains; then the benefits of instructing succeeding generations in how to make tools drove the emergence of language.

Evidence supporting this hypothesis began accumulating in the past decade as brain imaging found overlaps in the neural areas associated with language and those involved in tool use. More recently, groups led by Dietrich Stout, an archaeologist at Emory University in Atlanta, and Thierry Chaminade, a cognitive neuroscientist at Aix-Marseille University in Marseille, France, have taken to actually reproducing stone tools while tracking neural activity with positron emission tomography. In a series of experiments reported over the past 5 years, they showed that Oldowan toolmaking activates the left ventral premotor cortex, a region previously shown to be involved in both manual grip coordination and phonological processing. Late Acheulean tool production relies on those same regions, they found, plus other areas of the brain, including the inferior frontal gyrus, which is associated with abstraction and hierarchical organization (needed for executing subgoals along the way to a final product, for example), as well as larger scale discourse and language processing.

Follow-up experiments in which Bruce Bradley, an archaeologist at the University of Exeter in the United Kingdom, wore a data glove to record left hand finger movements suggested that it was the cognitive demands of making Acheulean tools—not left hand manipulation—that lit up the right brain.

The results “establish plausible evolutionary links” between specific toolmaking skills and language processing, Stout and Chaminade concluded in a review that appeared in *Philosophical Transactions of the Royal Society B* in January 2012.

But another step was needed to go from a language-ready brain to language. To see if teaching tool-

*Ninth International Conference on the Evolution of Language, Kyoto, 13–16 March. Tokyo Evolutionary Linguistics Forum, 19 March.

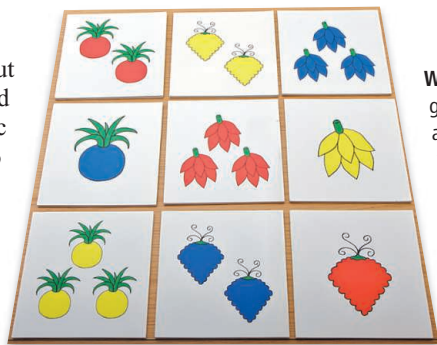
making had a role, Stout and colleagues used functional magnetic resonance imaging to capture brain activity of subjects as they watched an accomplished knapper at work. When observing Late Acheulean toolmaking, only those observers themselves skilled at toolmaking activated the intention-reading areas of the brain. They understood the ultimate goal of the craftsman, while the neophytes did not. The drive to bridge this gap in understanding “could have provided an adequate scaffold for the evolution of intentional vocal communication,” Stout and Chaminade wrote in their review.

Although neither Stout nor Chaminade attended the recent meetings, their work was at the center of many discussions. The co-opting of existing capabilities for new uses “is the way that evolutionary biologists typically explain major evolutionary innovations,” said evolutionary biologist Russell Gray of the University of Auckland in New Zealand during his talk. Gray and others gave tantalizing glimpses of work under way that builds on the Stout-Chaminade work to suggest that, among other things, the origins of abstraction and syntax might lie in tool-making pedagogy.

But not everyone was convinced. Massimo Piattelli-Palmarini, a biolinguist at the University of Arizona in Tucson, later told *Science* that a more genetic basis for language “will one day be discovered.” For now, evolution thinkers seem to have the upper hand.

The cultural factor

Those working on the cultural side of language evolution are also embracing experiments. Kirby argues that our modern use of language results from a dual inheritance. On one side, genetics gave us the cognitive and physiological capabilities for language. But language itself is passed from generation to generation by teaching and learning, a cultural process. “The language faculty evolved biologically, but languages themselves evolved culturally,” Kirby says. Kirby is particularly interested in the structure of language, specifically how meaningless syllables can be combined to make meaningful words, what he calls combinatoriality; and how words combine into phrases, compositionality. Thanks to these two properties, “I can produce sentences I’ve never said before and you’ve never heard before and yet you



Whatchamacallit? Randomly generated syllable strings acquired language-like properties as subjects used them to pick images from an array.

can understand me,” Kirby says.

Kirby and colleagues wanted to investigate how language acquired these structural aspects. Or, as his University of Edinburgh collaborator Hannah Cornish puts it, “What happens when you have some loose ideas of concepts and some ability to produce signals, but no preexisting system of combining these things together.” They hypothesized that the transmission of a language from generation to generation played a critical role. To test this idea, they recruited volunteers to learn a fictitious “alien” language.

(Calling it “alien” attracted participants.) Working at computer terminals, they were shown a series of words and the images they referred to. The words were actually randomly generated strings of syllables. Each of the images had a unique combination of color, shape, and patterning. The participants were then shown images and asked to type in the appropriate words. They were also asked to produce words for images with color, shape, and patterning combinations they hadn’t specifically learned.

The words as given by one participant were used to train the next in line, a process called iterated learning that resembles the cultural transmission of a language among generations.

Researchers tested different scenarios of that basic approach. In one, instead of individuals in each generation, there were pairs of participants who used the alien language to “communicate,” picking images from an array. (The pairs were separated and interacted via computer terminals so they could not point or gesture.) The words they recalled after the communication exercise were used to train the next pair in the chain. Other pairs simply did the communication task, again via computer terminals, over and over without the “language” being passed to a new generation.

When pairs of humans learned the words, used them to communicate, and then passed

them on through several generations, a compositional structure emerged. Parts of the words—the prefix, for example—consistently corresponded to color, and other parts became associated with shape or pattern. Succeeding pairs found the language progressively easier to learn and use accurately. Pairs at the ends of the chains could even recombine the parts of the words to accurately label images they had not specifically learned. When the language passed through a chain of individuals, thus skipping the communication step, it became ambiguous, with one “word” having multiple meanings. The pairs that worked just on the communication task eventually agreed on linking words with images, but the language remained an idiosyncratic pairing of syllables and meaning with no standardization or compositional structure. To get structural properties and improve learnability, “what is really crucial [is] a combination of naive



Tuning up. By passing through a succession of learners playing slide whistles, random notes became musical phrases.

learners and communication,” Kirby says.

Kirby admits that the linguistic structure they’re seeing may be an artifact from people who already have language. But Tessa Verhoef and Bart De Boer of the University of Amsterdam in the Netherlands, devised an experiment that avoided language altogether: Participants use slide whistles to produce whistling sounds unconnected to any meaning. The whistles produced by one participant were used to train the next. Again, structural elements—down-up and up-down whistles, repeated notes—emerged that were systematically reused and combined in various ways. And after several iterations, the whistles “become more learnable [and] more reproducible,” Verhoef says.

In addition to experiments with human subjects, Kirby and his colleagues ran computer simulations of populations acquiring language through iterated learning. Their computer model allowed them to test whether the tendency toward structure was likely to be innate—that is, genetically hard-wired—or the result of cultural transmission. Their work suggests that the structural properties seen in languages are more likely produced by the dynamics of cultural transmission. (The group's computer simulations were reported in the *Proceedings of the National Academy of Sciences* in 2007, and the results of the initial laboratory experiments appeared in the same journal in 2008. More recent work has yet to be published.)

The Kirby and Verhoef-De Boer experiments were “quite impressive and clever,” says Robert Van Valin, a linguist at Heinrich Heine University in Düsseldorf, Germany. That the computer simulations and human experiments agreed is significant, he says; it argues for the importance of cultural factors



Name that tune. Domesticated Bengalese finches (left) outsing wild white-rumped munias (right).

in the evolution of language. But there were still questions about what the experiments say about an ancient phenomenon. “It demonstrates one plausible path” for the emergence of language structure, says Sotaro Kita, a psychologist at the University of Birmingham in the United Kingdom.

Kirby says certain of his group's hypotheses are bolstered by other studies. They

have done some experiments using spoken words and have gotten the same results—with structure emerging after passage through several generations. Also, he says their experimental findings suggest that languages used by larger and more diverse groups, with more transmission to naïve learners, tend to be simpler. More complex languages appear to arise when user groups are smaller and more cohesive. That is consistent with what Gary Lupyan of the University of Wisconsin, Madison, and Rick Dale of the University of California, Merced, found in a survey of 2000 languages reported in *PLoS ONE* in 2010. “The analyses suggest that languages spoken by large groups have simpler inflectional morphology than languages spoken by smaller groups as measured on a variety of factors,” the pair wrote. Like organisms, language structures appear to adapt to their environment.

More evidence that complex language arises in close-knit, stable communities comes from a study of songbirds presented at the Tokyo Evolutionary Linguistics Forum by biopsychologist Kazuo Okanoya of the University of Tokyo. He reported that long-domesticated Bengalese finches have much more complex songs than their close cousins that live in the wild, white-rumped munias. Okanoya says that in the wild, the song needs to be simple and distinct so females can find males of their own species. But in a birdcage full of Bengalese finches, females take mastery of a complex song as a sign of male fitness. “Domestication freed songs from the function of species identity and female choice promoted complexity in Bengalese finches,” Okanoya concludes in a paper now in press at *Interaction Studies*. By extension, Okanoya says human self-domestication could have set the stage for human language to gain complexity.

No one line of investigation is going to answer all the questions, Kirby says. Understanding the evolution of language “requires a convergence of evidence from an extraordinarily diverse set of disciplines,” he says.

That conciliatory tone echoed throughout Evolang9. People spoke of fitting together the pieces of a very complex puzzle. A new generation of language researchers “is more interested in an interdisciplinary approach and more tolerant of complexity,” says Rafael Núñez, a cognitive scientist at the University of California, San Diego. That bodes well for the Evolang conferences, which were founded on the notion that all those studying language evolution should have something to say to one another.

—DENNIS NORMILE

Where Time Goes Up and Down

In Western cultures, the future lies ahead; the past is behind us. These notions are embedded in both gestures and spoken metaphors (looking forward to next year or back over the past year). A forward hand motion typically accompanies talk of the future; references to the past often bring a wave over the shoulder.

It is hard for most Westerners to conceive of other ways of conceptualizing time. But in 2006, Rafael Núñez, a cognitive scientist at the University of California, San Diego, reported that for the Aymara, an ethnic group of about 2 million people living in the Andean highlands, in both spoken and gestural terms, the future is unseen and conceived as being behind the speaker; the past, since it has been witnessed, is in front. They point behind themselves when discussing the future. And when talking about the past, Aymara gesture farther in front of them the more distant the event (*Science*, 23 June 2006, p. 1723).

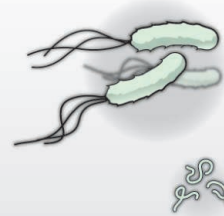


Heads up. This Yupno man of Papua New Guinea points downhill when speaking of the past, whether facing uphill (left) or downhill (right).

ing—and gesturing—about time: The Yupno people, who inhabit a remote valley in Papua New Guinea, think of time topographically. No matter which way a speaker is facing, he or she will gesture uphill when discussing the future and point downhill when talking about the past. “It can only occur in small societies that share an ecological niche,” Núñez says of their finding, in press at *Cognition*. These different abstractions of time, including gestures, indicate the importance of the cultural aspects of language evolution, he contends.

Núñez’s “very interesting” Yupno study “provides a tiny glimpse into the way one group of people relates to their world linguistically and cognitively,” says Erica Cartmill, a psychologist at the University of Chicago in Illinois who studies the use of gesture by great apes and children. She says more such comparative studies would help clarify the relationship between gesture, speech, and cognition in different cultures.

—D.N.



LETTERS

edited by Jennifer Sills

Chile's Research Planning Falls Short

FOR YEARS, CHILE'S POLITICIANS AND ECONOMISTS HAVE TALKED ABOUT THE NEED to increase scientific research to become a developed nation. However, indicators of governmental performance and research policy in Chile, especially when compared with other Organization for Economic Co-operation and Development (OECD) countries—an organization that Chile has only recently joined—indicate poor performance in terms of

investment, researchers, and even public promotion and attitudes to research (1–3). In recent years, Chile's scientific community has faced possible cuts in research funding, and the country's graduate students have been exposed to cuts or delays in calls for funding, publication of results about who will receive funding, and even delivery of the associated funding, including essential fellowships and stipends for their professional development.

Politicians, researchers, and even international organizations have criticized Chile's lack of a modern and appropriate government policy for research (4–7).

Demand for change. Graduate students from Chilean universities protest for improvements in the management of the Human Capital Program from the government.

The governmental institution responsible for research administration, CONICYT, was created more than 40 years ago, and since then it has been subjected to successive modifications that undermined its autonomy and relevance. For example, in 1973, the Scientific Advisory Council from CONICYT was eliminated and its attributions were transferred to the President of CONICYT (8). The reinstatement of this Council, which would allow direct communication and advice from the scientific community, remains an urgent need (9).

In contrast, Argentina and Brazil have Ministries of Science and Technology. Peru is also working on the creation of a new Ministry of Science, Technology, and Innovation (10). They are not alone; more than half of the countries in the world, including more than 20 countries from the OECD, have a Ministry or Minister for Science (11).

Chile desperately needs to update its national planning for research. We agree with the recommendations of the experts who advise the creation of a Ministry of Science and Technology (4–7). The Ministry should define a new national plan for science and development [the current plan hails from 1988, among the oldest in South America (12)]; facilitate communication between universities, research centers, and industries; improve public management and funding of national scholarship programs; and engage citizens on the value of scientific research.

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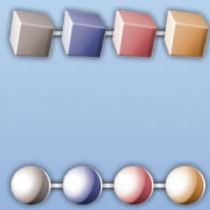
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Don't Jump to Conclusions on Fraud

IN THEIR OTHERWISE FINE EDITORIAL "Addressing scientific fraud" (2 December 2011, p. 1182), J. Crocker and M. L. Cooper use unfortunate and ill-advised language when they refer to "the fraudulent work published" by Marc Hauser. Crocker and Cooper rightly criticize Harvard University for keeping secret its findings in the investigation of Hauser. However, stating unreservedly that Hauser has committed fraud is not acceptable. First, all that Harvard has stated is that



Designer amino
acids and polymers

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CORRECTIONS AND CLARIFICATIONS

Review: "Natural SIV hosts: Showing AIDS the door" by A. Chahroudi *et al.* (9 March, p. 1188). On page 1192, middle column, second full paragraph, the sentence that reads "The SIV_{smm} and SIV_{mac} (and SIV_{agn} and HIV-2, but not SIV_{mnd-1} and HIV-1) viruses express Vpx that antagonizes the newly described SAMHD1 host restriction factor..." is incorrect. Instead, it should read: "The SIV_{smm} and SIV_{mac} (and SIV_{mnd-2} and HIV-2, but not SIV_{agn} , SIV_{mnd-1} , and HIV-1) viruses express Vpx that antagonizes the newly described SAMHD1 host restriction factor..."

Perspectives: "Autophagy in tumor immunity" by R. K. Amaravadi (16 December 2011, p. 1501). The term "allograft" was misused to refer to the animal models described in Michaud *et al.* (2) and Noman *et al.* (4). In both papers, syngeneic transplantable tumor models were used as hosts for evaluating the immune responses.

TECHNICAL COMMENT ABSTRACTS

Comment on "Universality in the Evolution of Orientation Columns in the Visual Cortex"

Yicong Meng, Shigeru Tanaka, Chi-Sang Poon

Kaschube *et al.* (Reports, 19 November 2010, p. 1113) argue that pinwheel density in three mammalian species follows a universal constant of π as predicted by their orientation-selective suppressive long-range connectivity model. We dispute their conclusions and suggest that a simple brain size–pinwheel density scaling law suffices in predicting the self-organized and disorganized orientation maps from primates to rodents.

Full text at www.sciencemag.org/cgi/content/full/336/6080/413-c

Response to Comment on "Universality in the Evolution of Orientation Columns in the Visual Cortex"

Wolfgang Keil, Matthias Kaschube, Michael Schnabel, Zoltan F. Kisvarday, Siegrid Löwel, David M. Coppola, Leonard E. White, Fred Wolf

Meng *et al.* conjecture that pinwheel density scales with body and brain size. Our data, spanning a 40-fold range of body sizes in Laurasiatheria and Euarchonta, do not support this conclusion. The noncolumnar layout in Glires also appears size-insensitive. Thus, body and brain size may be understood as a constraint on the evolution of visual cortical circuitry, but not as a determining factor.

Full text at www.sciencemag.org/cgi/content/full/336/6080/413-d

Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the past 3 months or matters of general interest. Letters are not acknowledged upon receipt. Whether published in full or in part, Letters are subject to editing for clarity and space. Letters submitted, published, or posted elsewhere, in print or online, will be disqualified. To submit a Letter, go to www.submit2science.org.

its secret committee found that Hauser has engaged in unspecified types of "scientific misconduct" (1). Certainly, there are many types of misconduct that do not constitute fraud. Second, Harvard's findings are neither public nor the result of legal review and thus are not definitive. Finally, recent developments call into question the severity of whatever it is that Hauser may have done: Two of the three published studies in question have been repeated, and the results have confirmed the originally published work (2–4). Consequently, scientific fraud does not seem to be the issue involved in these two cases. One has to wonder what the problem was in these and the other cases in question (5–6).

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Finding Balance in Fisheries Management

S. M. GARCIA *ET AL.* ("RECONSIDERING THE consequences of selective fisheries," Policy Forum, 2 March, p. 1045) presented a valuable framework for considering how balanced fisheries harvest could improve ecosystem status. However, balanced harvest has some shortfalls as a practical policy, particularly in terms of economic realities and as a method of ecosystem-based management (EBM). Fisheries catch is a function of supply and demand, and most gear types and fishing

efforts target high-value resources (1). Until demand exists for high-biomass and low-price species, a truly balanced fishery is not feasible without subsidies (2) [but see (3)].

In addition, true EBM requires not just balanced fishing, but balanced management of risk across multiple sectors [e.g., integrated ecosystem assessments (4)] to ensure population viability for all species and healthy ecosystems (5, 6). Although Garcia *et al.* considered fishing effects across a suite of fished species, the balanced harvest concept does not factor in fisheries' independent ecosystem needs, such as forage resources for other predators (seabirds, for example) (7).

Furthermore, there will always be a need to protect long-lived, slow-maturing, low fecundity species that are targeted or affected by fishing (8). Marine species experience cumulative impacts and face mortality risks beyond fisheries, and these sources often act disproportionately on specific ecosystem components and life history stages (5, 9). Cumulative impacts can reduce the abundance of these species in the ecosystem to a level that can withstand minimal to no fisheries harvest, necessitating selective fishing.

True EBM requires not just balanced fishing, but balanced management of risk across multiple sectors to ensure population viability for all species and healthy ecosystems.

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Comment on “Universality in the Evolution of Orientation Columns in the Visual Cortex”

Yicong Meng,¹ Shigeru Tanaka,² Chi-Sang Poon^{1*}

Kaschube *et al.* (Reports, 19 November 2010, p. 1113) argue that pinwheel density in three mammalian species follows a universal constant of π as predicted by their orientation-selective suppressive long-range connectivity model. We dispute their conclusions and suggest that a simple brain size–pinwheel density scaling law suffices in predicting the self-organized and disorganized orientation maps from primates to rodents.

Kaschube *et al.* (1) proposed that the emergence of orientation columns in visual cortex can be explained by a common design in a self-organized network with suppressive long-range connectivity. Based largely on theoretical grounds under a set of assumptions, their model predicts that the pinwheel density, defined as the average number of pinwheels per orientation-hypercolumn area, approaches a universal constant of π . Kaschube *et al.* reported that the precisely measured pinwheel densities in three mammalian species were virtually identical and approached the predicted value of π . These findings, if true, would be of fundamental importance in implicating a universal law in the self-organization of orientation columns across the mammalian phylum. However, several concerns arise that cast doubts on the validity of the proposed universal constant.

The notion of a common design for orientation maps across different mammalian species fundamentally runs counter to a classic animal scaling law, which states that many behavioral and physiological attributes scale in proportion to brain sizes (2). To test whether pinwheel density also obeys this scaling law or is constant at π , it is imperative to examine species with widely different brain sizes. Unfortunately, all three species studied by Kaschube *et al.* (ferret, galago, and tree shrew) are of similar sizes for primary visual cortex. While emphasizing the phylogenetic and ecologic diversity of these chosen species, the authors have unwittingly limited their data to a single point on the brain size scale (Fig. 1). Could the pinwheel density depart from the proposed constant value of π for much larger or smaller species? Interestingly, extant data in the literature suggest that absolute pinwheel density (number of pinwheels per mm^2) may vary widely

across different species (3–9). As summarized in Table 1, which is modified from a previous report by the same group (3), the pinwheel densities calculated from reported data for ferret and tree shrew consistently underestimate the value of π , whereas those for much larger species, such as macaque monkey, overestimate it. These estimates from early studies are necessarily imprecise because of the limitations of such measurements. Despite this, the remarkable correlation between the estimated pinwheel densities and sizes of primary visual cortex across a wide range of species (see Fig. 1) cannot be attributed to experimental

errors and data variability alone, because any systematic or random errors would equally apply to studies in all animal species. The overestimation of the value of π in macaque is particularly revealing in light of the underestimation in ferret and tree shrew. In contrast, the authors’ decidedly precise measurements of pinwheel densities in three species with similar brain sizes cannot answer this very basic question about the effect of brain size scaling. To substantiate the proposed common design for orientation maps, the *onus probandi* is to demonstrate that the same universal constant of π for pinwheel density holds regardless of the brain sizes of the specific species chosen.

Whereas definitive data for pinwheel density in larger and widely studied species such as cat and monkey showing unequivocal conformance to the proposed universal constant of π remain to be seen, the most telling evidence to date that pinwheel density may scale with brain sizes rather than being fixed at π comes from much smaller species such as small rodents (rats and mice), in which the primary visual cortex is one order of magnitude smaller than those of ferret and tree shrew (10, 11). It is well known that the visual cortex of rats and mice has a random salt-and-pepper arrangement of preferred orientations (12). Hence, the layout of orientation maps, if any, is totally fractured, and pinwheel density is virtually nil in these small animals (Fig. 1). Thus,

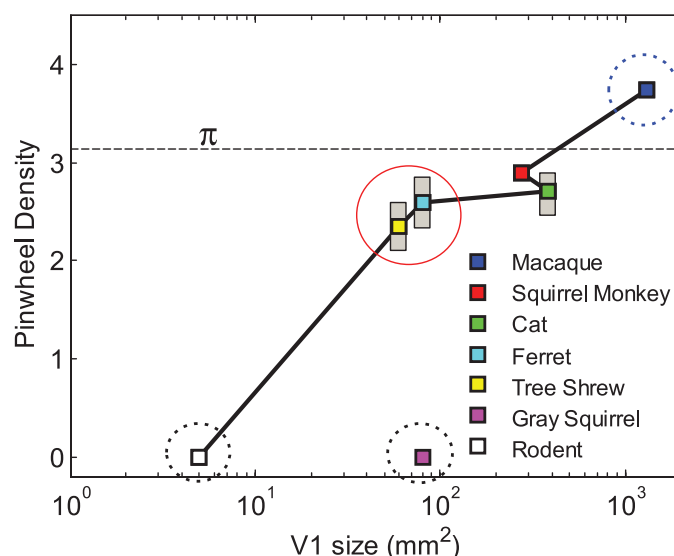


Fig. 1. Pinwheel densities (filled squares) versus primary visual cortex (V1) areas for different species. Filled squares give the mean pinwheel densities, and gray bars show the upper and lower range of the pinwheel densities estimated from different data sources listed in Table 1. The solid circle (in red) indicates two species (ferret and tree shrew) studied by Kaschube *et al.*, both of which prove to be of similar V1 sizes (data for the V1 size of galago not available, although its body size is similar to that of ferret and tree shrew). Dashed circles indicate species with one order of magnitude larger (macaque monkey), similar (gray squirrel), and one order of magnitude smaller (mouse) V1 areas compared with species studied by Kaschube *et al.* The horizontal dashed line gives the reference level of π . Pinwheel density of macaque monkey overestimates the critical value of π , whereas those of ferret and tree shrew underestimate it. Such V1 size-dependent correlation cannot be attributed to systematic or random measurement errors alone. The pinwheel densities plotted in this figure are from Table 1. The V1 sizes are from (10, 11).

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Table 1. Pinwheel density in different species. [Modified from (3)]

Species	Pinwheels per mm ²	Wavelength (mm)	Pinwheel density
Macaque monkey	8.1 (5)	0.68	3.75
Squirrel monkey	11.1 (5)	0.51	2.9
Cat	2.1 (4), 2.4 (6), 3.4 (7), 3.5 (8), 2.6 (9)	1.1 (4, 6), 0.85 (7, 8)	2.54, 2.9, 2.46, 2.53, 3.1
Ferret	5.5 (6), 4.5 (8)	0.72	2.85, 2.33
Tree shrew	7.9–9.5 (3)	0.52	2.1, 2.6
Rodent	0	N/A	0

size does matter. Apparently, the size of the visual cortex is not the only factor that may influence pinwheel density, because orientation maps are also absent in some larger rodents such as gray squirrel (10) and some lagomorphs such as rabbit (13) (Fig. 1). It has been suggested that differences in intracortical circuits may explain such anomalies (11, 12), further arguing against a common design in these circuits.

Kaschube *et al.* draw questionable conclusions based on experimental data from a singular subset of species, with limited applicability to the prediction of pinwheel densities across the mammalian phylum. We believe that a simple scaling law for brain size–pinwheel density correlation suffices in most cases in predicting the self-organized and disorganized orientation maps from primates to rodents.

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Response to Comment on “Universality in the Evolution of Orientation Columns in the Visual Cortex”

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Meng *et al.* conjecture that pinwheel density scales with body and brain size. Our data, spanning a 40-fold range of body sizes in Laurasiatheria and Euarchonta, do not support this conclusion. The noncolumnar layout in Glires also appears size-insensitive. Thus, body and brain size may be understood as a constraint on the evolution of visual cortical circuitry, but not as a determining factor.

We presented a comparative study of visual cortical orientation columns and pinwheels in three mammalian species whose evolutionary paths separated more than 65 million years ago (*I*). For this purpose, we introduced methods to measure pinwheel density objectively—i.e., insensitive of image data pre-processing [*I*] and supporting online material (SOM) of (*I*), pp. 3–12 and 29–41]. We found that statistical measures characterizing the spatial layout of pinwheels from the scale of individual hypercolumns to the entire primary visual cortex (V1) were virtually identical, agreeing with an accuracy of a few percent. To understand how distinct evolutionary lineages can independently evolve this common design, we examined a broad set of mathematical models for the developmental self-organization of orientation columns [*I*] and SOM of (*I*), pp. 13–62]. We found that models from a symmetry-defined class, exhibiting a universal (i.e., model-independent) solution set, robustly predict every aspect of the common design when suppressive long-range interactions are dominant [*I*] and SOM of (*I*),

pp. 13–41]. This suggests that developmental network self-organization has canalized the evolution of neuronal circuitry underlying orientation maps in these species into the common design. A predicted signature of this mechanism is a pinwheel density close to the mathematical constant π . Confirming this prediction, we found that mean pinwheel density was indeed statistically indistinguishable from π ($\pm 2\%$).

Meng *et al.* claim that pinwheel density is not an invariant but a function of V1 size (2). They conjecture a pinwheel density scaling law that can be approximated by

$$\rho = 3/2 \log_{10}(A/3 \text{ mm}^2)$$

where A is V1 surface area [figure 1 in (2)]. Their scaling law predicts substantial differences between ferrets/tupaia, galagos, and cats. With 80 mm², 200 mm², and 450 mm² V1 size, respectively (3, 4), pinwheel density in cats (galagos) should be 53% (28%) larger than in ferrets/tupaia. Our methods to accurately estimate pinwheel density are well suited to test this prediction. Figure 1A presents measured pinwheel densities for the four species graphed against body weight. Pinwheel densities appear invariant with respect to body weight. The hypothesis that mean pinwheel density in cat (galago) is more than 50% (20%) larger than in ferrets can be rejected with virtual certainty [$P < 0.0001$ (0.0001), bootstrap test]. Meng *et al.* suggest greater variation in pinwheel density across species; however, those data were derived from studies with small sample sizes using first-generation optical imaging methods that are subject to various systematic errors described in detail in [SOM of (*I*), figures S2 and S3 and pp. 3–12]. Our analysis unambiguously indicates that pinwheel density is a genuine invariant feature of orientation column layout over a wide range of V1 sizes and that it is insensitive to hypercolumn size (Fig. 1, B and C).

Figure 1D presents V1 design type for Laurasiatheria, Euarchonta, and Glires varying

in body weight over 5 orders of magnitude. As this display suggests, the interspersed, “salt-and-pepper” layout observed in rodent and lagomorph (Glires) V1 (3, 5) provides no evidence for a size-dependent V1 design. Interspersed layouts constitute a qualitatively distinct design type [for further discussion, see (*I*), p. 1115] from that observed in Laurasiatheria and Euarchonta. Glires exhibit this layout for V1 sizes that vary from 3 to 80 mm², irrespective of ecological niche and visual behavior (3). Indeed, Glires cortex generally deviates in cellular composition from primate or carnivore cortex (6, 7) and exhibits interspersed organization also for direction selectivity in primary somatosensory cortex and characteristic frequency in primary auditory cortex (8, 9). Thus, the interspersed layout is a putative Glires-typical trait.

However, the functional organization of V1 must not be imagined to be completely independent of area size. Orientation hypercolumns have a spatial extent on the order of a millimeter, with hypercolumn areas ranging between 0.4 mm² and 1.4 mm². For species in which V1 size approaches this range, it becomes hard to conceive of V1 as being organized by a system of orientation columns. A size constraint prohibiting a conventional system of orientation columns should, for instance, apply to the V1 of eurasian shrews (*Suncus etruscus*), which is confined to a mere 0.2 mm² of visual cortex (10). It also appears plausible for the last common ancestor of carnivores and primates, called the boreoeutherian ancestor [SOM of (*I*), pp. 63–64]. Its closest living relative, the tenrec (*Echinops telfairi*), weighs about 100 g and has a visual cortex that totals 2 mm² (11). Fossils of stem eutherians [*Asioryctes nemegetensis*, 43 g, 0.5 ml endocranial volume (EV); *Kennalestes gobiensis*, 39 g, 0.3 ml EV; *Zalambdalestes lechei*, 83 g, 1 ml EV] indicate even smaller body and brain sizes (11, 12) (Fig. 1E). If the boreoeutherian ancestor in fact lacked a system of orientation columns due to such a size constraint, then larger visual cortices exhibiting orientation columns must have independently emerged in the evolutionary lineages leading to extant primates, tree shrews, and carnivores. In any event, as indicated in Fig. 1E, visual cortical organization has been challenged—among other changes—by a dramatic increase of animal and, consequently, V1 size in the wake of the Cretaceous-Tertiary (K-T) extinction event 65 million years ago [for further discussion, see the SOM of (*I*), pp. 63–64]. In view of these aspects, it seems remarkable that lineages separated over the entire evolutionary history of boreoeutherian mammals precisely follow a common design.

In summary, studying animals of different V1 size confirmed pinwheel density as a genuine invariant of orientation map design. The interspersed organization found in Glires constitutes a distinct design type also insensitive to V1 size. Orientation columns so far have only been found in large visual areas and may not exist in very

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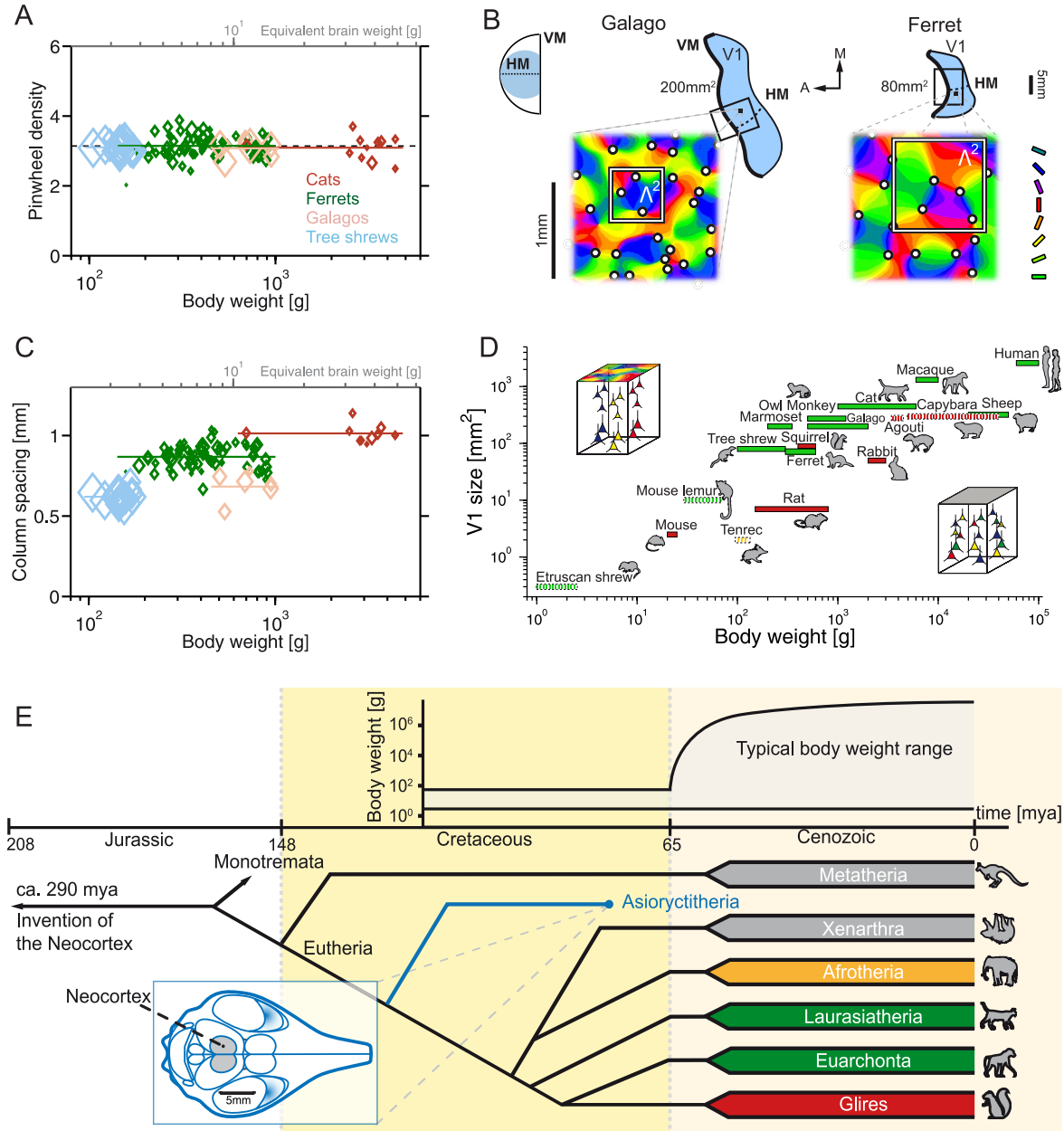


Fig. 1. Brain size, V1 surface area, and pinwheel density in the evolution of visual cortical organization. **(A)** Pinwheel density is size invariant. Dimensionless pinwheel densities ρ versus body size m_{body} in tree shrew, galago, ferret, and cat. Tree shrew, $\rho = 3.11$ (3.05, 3.16), mean and 95% confidence interval (CI), $n = 20$ hemispheres (hem.); galago, $\rho = 3.07$ (2.87, 3.23), $n = 6$ hem.; ferret, $\rho = 3.15$ (3.07, 3.22), $n = 70$ hem.; cat, $\rho = 3.09$ (2.89, 3.29), $n = 11$ hem.; symbol size proportional to size of the measured region in units of one hypercolumn Λ^2 . Solid lines indicate average pinwheel density in each species. Black dashed line is at $\rho = \pi$. Linear regression $\rho = \rho_0 + \rho_1 \log_{10}(m_{\text{body}}/\text{g})$ estimates a slope $\rho_1 = -0.02$ ($-0.14, 0.11$), not significantly different from zero. Top axis indicates equivalent brain weight (13). **(B)** Galago and ferret differ strongly in surface area and hypercolumn size but have indistinguishable pinwheel density. (Top) V1 surface area and representation of the visual hemifield in galago (left) and ferret (right). Blue area, V1; HM, horizontal meridian; VM, vertical meridian. (Bottom) Typical orientation map insets in galago (left) and ferret (right); black box identifies typical imaging field in experiments. White dots, pinwheels; white square, area of one hypercolumn. Galago V1 contains more than 400 orientation hypercolumns, ferret V1 only about 100. **(C)** Orientation column spacings versus body weights. Orientation column spacing exhibits substantial interspecies differences. Solid lines indicate

average column spacing in each species. Tree shrew, $\Lambda = 0.62$ mm (0.61, 0.63), $n = 20$ hem.; galago, $\Lambda = 0.68$ mm (0.62, 0.74), $n = 6$ hem.; ferret, $\Lambda = 0.87$ mm (0.85, 0.88), $n = 70$ hem.; cat, $\Lambda = 1.01$ mm (0.99, 1.04), $n = 11$ hem.; symbol size as in (A). **(D)** Both columnar and interspersed arrangements of orientation preference appear over wide and overlapping ranges of V1 surface area and body weight. The two design types are indicated by schemes: upper scheme, columnar; lower scheme, interspersed. Green color indicates species of the Laurasiatheria or Euarchonta clade. Red color indicates Glires species. Filled/hatched bars indicate known/unknown spatial organization of orientation preference. Green filled bars indicate columnar, red filled bars indicate interspersed organization. [Data from (3, 4, 10, 14, 15)] **(E)** Mesozoic and cenozoic macroevolution of extant mammals showing divergence into six major clades: Monotremes, Marsupials, Afrotheria, Xenarthra, Laurasiatheria, and Euarchontoglires, which split into Euarchonta and Glires (11). Eutherian size expansion after the K-T extinction event is indicated on top. Large V1 architecture independently evolved at least six times during the radiation of extant mammals. The anatomical scheme depicts the cranium of a representative late cretaceous stem eutherian (Asioryctitherian). This close relative to the last common ancestor of extant Eutheria had a small V1, presumably lacking orientation columns [SOM of (1), pp. 63–64].

small brains such as those of many late cretaceous mammals. In their descendants, the K-T extinction event enabled an explosive increase in body and brain size millions of years after the major mammalian lineages had separated. It triggered the separate evolution of architectures for large visual areas in distinct lineages. Our theory of universality in network self-organization explains how they could independently develop a common design. It is the only known explanation for the quantitatively precise agreement of orientation column layouts in tree shrew, galago, ferret, and cat. Other mammalian lineages are predicted to adopt the same design when using qualitatively similar developmental mechanisms. Precise quantification of visual cortical architecture in mammals from the extremes of body and brain sizes in all therian clades will help to clarify this fascinating chapter of brain evolution with rigor and certainty.

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FILM: ENVIRONMENT

Wildlife, Wildlands, Water, and More

The Environmental Film Festival in the Nation's Capital was launched in 1993 to highlight works that advance "environmental understanding through the power of film." The 20th festival brought 180 films from 42 countries to Washington, D.C., this March. Most of these documentaries, stories, animations, and children's films were recent releases. Many of them explored the complex links connecting the environment with our health or foods. The festival's website (www.dcenvironmentalfilmfest.org) includes sketches of all of this year's offerings; here our reviews reflect on nine of them.



Wild Scandinavia: Finland. Oliver Goetzl and Ivo Nörenberg, directors. Gulo Film Productions, Germany, 2011. 52 minutes.

Following the seasons through a year in Finland, this portrait of life in northern wilderness intertwines stories of brown bears, flying squirrels, ducks, wolverines, and black woodpeckers as it records mating, nesting, birth and training of the young, and predation. The film begins in mid-March, which still looks like deep winter to those of us in more temperate climes. Crisp views of the snow-covered landscapes convey their beauty. Time-lapse footage of the aurora borealis shows the spectacular displays typical of late winter, and low-altitude aerial photography captures the mosaic of lakes, rivers, marshes, and coniferous forest that carpets this land of modest hills. As the seasons progress, slow-motion photography records the defense of mating territory by the red-throated loon, whose haunting calls evoke deep, dark



woods. Close-ups within a recycled woodpecker hole reveal hatching and fledging of goldeneye ducklings. An osprey finds fish and then returns to its nest, where the chicks agreeably wait their turn for bites from mother. With cameo appearances by wild forest reindeer, ravens, owls, and the usually nocturnal lynx, the film documents the mutual interactions that sustain the ecosystem. The narrator reminds us of losses attributed to human civilization: Although 80% of Finland is covered with trees, only 3% of the ancient coniferous forests remain. Wolverines have dwindled to about 150, there are but 260 Saimaa ringed seal left in the wild, wild forest reindeer are found only in two small portions of Lapland, and wolves, still subject to legal hunting, now number only 150. After a first snowfall sparkles on the trees in diminishing light, winter arrives with a blizzard. Wolves, ravens, and a bear compete over a kill, which this time goes to the wolves. —Pamela J. Hines

The Tsunami and the Cherry Blossom. Lucy Walker, director. Supply and Demand Integrated, USA, 2011. 40 minutes. <http://thetsunamiandthecherryblossom.com>.

The annual blooming of the cherry trees in Japan signals the arrival of spring and the new beginnings that come with it. But when such beauty appears in the wake of one of the largest natural disasters in the country's history, it is a



sad reminder that destruction is also a recurring force in nature.

Lucy Walker's short documentary *The Tsunami and the Cherry Blossom* begins with haunting hand-held footage shot from atop a bluff in Miyagi prefecture of the swelling tsunami caused by the 2011 magnitude-9.0 Tohoku-oki earthquake. After the camera captures the unbelievable sight of vehicles and entire buildings sweeping across the valley below—with the panicked reactions of incredulous fellow observers audible in the background—the screen goes black just as we see raging water approaching a few remaining, fleeing residents and rescuers. When cameras return to the site of the opening scene just weeks later, this time with Walker and her small crew in tow, we see a destroyed city in the background, framed softly by the familiar sight of budding cherry blossoms.

Walker interviews several survivors who were willing to tell their chilling stories of survival and loss. She also includes stories of individuals and communities rallying together and people helping each other cope and recover as they grieve. As the film's focus shifts in and out of the relationship that the Japanese people have with cherry blossoms, we see how these beautiful yet ephemeral blooms provide them with a symbol of hope and strength. One resident remarked, as he was searching through the rubble of his home: "The plants are hanging in there, so us humans had better do it too."

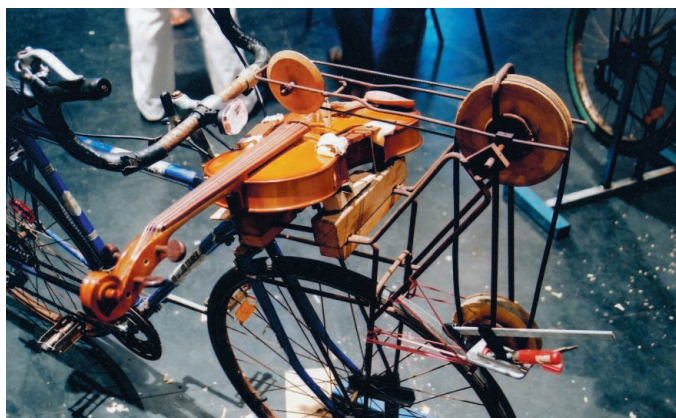
Natural disasters of such an extreme scale and magnitude are extremely difficult to grasp, even for those who experience them first-hand. Walker conveys how hopeless and lost many survivors felt in the days and weeks after what was to many a tsunami of unfathomable size. Through grim yet beautiful footage of several leveled cities and villages, the film also shows how we remain vulnerable to the powers of nature even in a well-prepared country like Japan. Nonetheless, the photography, the survivors, and the cherry blossoms themselves remind viewers that resiliency and renewal are also important forces of nature—even in the aftermath of destruction.

—Nicholas S. Wigginton

The Reach of Resonance. Steve Elkins, director. Candela Films, USA, 2010. 118 minutes. www.reachofresonance.com.

Have you ever heard Earth's magnetic field as melody or listened to a plant as part of a musical ensemble? What would an urban riot sound like if transcribed for string quartet? Can a piece of music be defined by geographic rather than chronologic length? In *The Reach of Resonance*, Steve Elkins considers the meaning of music and how it relates to its surrounding environment through the work of four avant-garde musicians: John Luther Adams, Miya Masaoka, Bob Ostertag, and Jon Rose. The film offers a wide spectrum of aural experiences and thought-provoking explorations of the making of environments of sound. Some of the featured creations seem the essence of conceptual art, with the final aesthetics taking a backseat to the thought processes behind the work. But others, including pieces performed by the world-renowned Kronos Quartet, are evocative in their beauty.

The film takes a risk by first plunging into the esoteric work of Rose, someone whose intent seems at times as much to annoy others as to explore the possibilities of sound. He brags about his "sonic terrorism" and conducts



much of his interviews from underneath the covers of (presumably) his bed. However, his thoughtfulness about music cuts through the self-indulgence, and his ongoing project of playing fences as instruments succeeds in making us think about a broader environment and how it intersects with the world of art.

Audiences will find a little bit of everything here, including compositions performed by Madagascar hissing cockroaches crawling over Masaoka's naked body, transcriptions by Adams from the seismic activity of inner Alaska, and samplings drawn by Ostertag from a young El Salvadoran boy's graveside speech about his father. Some may take exception at what is described as music—as opposed to sound—and Elkins's presentation does falter somewhat when it veers too far or long into political or historical discourse. But through its examination of the role of environment in the creation of music, and the deeper exploration of the environments created by these sounds, the film achieves its goal of finding meaning in music beyond mere aesthetic pleasure.

—Yael Fitzpatrick

Radioactive Wolves. Klaus Feichtenberger, director. ORF/EPO-Films, Austria, 2011. 50 minutes.

Twenty-six years ago, on 26 April 1986, reactor number four at the Chernobyl power plant in the Ukraine ignited. This, the largest nuclear accident in history, released a radioactive plume that drifted across Europe. The catastrophe eventually led to the displacement of over 300,000 people from the areas around the plant (including the entire city of Prypiat) and the creation of a 30-km-radius human-exclusion zone. Initial responses of wildlife to the accident mirrored concerns about human health, including immediate mortality and subsequent deformities in newly born young. However, the removal of people from the actively guarded zone has, by default, estab-

lished effective wilderness in a region that was long dominated by human activities. Filmmaker Klaus Feichtenberger's *Radioactive Wolves* demonstrates that keeping people out of a landscape, even one as heavily damaged as this zone, has the power to rapidly and profoundly restore wildness.

Feichtenberger and his crew were only permitted to enter the zone for eight hours at a time. During their visits, they followed local researchers who are attempting to determine the size of the zone's wolf population and whether its birthrate is sufficient to sustain it. The film includes many striking images, and there is something extremely satisfying about watching previously persecuted species—such as wolves, white-tailed eagles, reintroduced European bison and Przewalski's horses, and 2.5-meter-long catfish—moving through a "rewilded" landscape. When a young wolf is captured to be radio-collared, however, a Geiger counter held above the anesthetized animal screams, registering high levels of radiation and reminding us that this unintended wildlife sanctuary comes with a price. Nonetheless, as the film leads us through the discovery of newly born wolf litters to the conclusion that the local wolf population is healthy and self-generating, we also see a landscape being reborn due to freedom from human pressures and impact. This is refreshing, as we inhabit a world where humans increasingly dominate nearly every environment. Very little scientific research has been conducted on wildlife populations in the exclusion zone, so there is reason to be cautious about interpreting the film's scenes as definite indicators that the exclusion's overall impact on wildlife has been positive. Still, it is hard to ignore the impressions of restoration and hope that the film imparts. In the end, the transition from tragedy to rebirth suggests a resilience in nature that many have begun to doubt but that requires we provide the space for recovery.

—Sacha Vignieri

Platypus in the Tropics. Alberto Vale, director. WildCAM Australia, Australia, 2012. 40 minutes. www.wildcamaustralia.com/platypus_in_the_tropics.html.

This charming documentary chronicles a year, beginning in May, in the life of a few platypuses inhabiting a tropical rainforest in northern Queensland, Australia. Northern platypuses are smaller than their southern conspecifics, and, unlike those, they remain active year-round. May is part of the dry season and a good time to observe platypuses. While we watch the engaging creatures dive and dart with surprising agility, the narrator describes various physiological and ecological traits: e.g., the electromechanical receptors on the bill that help the animals locate small crustaceans and other invertebrate prey, the very dense fur coat that keeps internal temperature close to 32°C,



and the approximate range (about half a kilometer) and number of burrows (six to eight) of a single animal. August brings male aggression in advance of mating, and females begin inspecting burrows. By September, females are gathering materials for their selected nests. Filmmaker Alberto Vale

buried cameras inside platypus tunnels and burrows. His patience rewards us with views of a female incubating her eggs and the subsequent appearance of small, naked, 2-week-old young. The females are recorded feeding their young about once a day; they plug their nests when they leave. After about four months, the juveniles emerge to take their first swim, mastering the basics within 24 to 48 hours. January is part of the rainy season, and particularly heavy rains or poorly located burrows can cause young platypuses to drown in their nests. Young can also be swept to their deaths by fast currents.

Platypus in the Tropics has a retro feel. There are no cross-continental journeys, bloody combats, or environmental disasters. Vale wisely lets the platypus's endearingly odd appearance and behavior win over viewers. Seven years of filming yielded ample footage of platypuses swimming, foraging, and burrowing, but the film's highlights are the glimpses into the nest with its young.

—Trista Wagoner

The Man Who Stopped the Desert. Mark Dodd, director. 1080 Films, UK, 2010. 62 minutes. www.1080films.co.uk/Yacoubamovie. Mark Dodd was a cameraman for the BBC when he met Yacouba Sawadogo, a farmer in northern Burkina Faso. He was so impressed and inspired that he quit his job and formed his own company so that he could make a film about Yacouba.

Yacouba's farm lies in the Sahel, a wide belt across Africa past the Sahara's southern edge but before the lush grasslands begin. Between 1975 and 1985, the region suffered from a severe drought, and many families left their homes to seek food and work in cities. Looking for a solution, Yacouba thought of modifying a traditional farming technique locally known as *zai* (pits). He began digging pits during the dry season, making them much wider and deeper than customary and filling the holes with compost as well as seeds. His *zai* retained more water than traditional versions, and his crops flourished. Unlike nearly all other local farmers, he was able to feed his family with his harvest.

Realizing the importance of trees to wind and soil, Yacouba also began a forest on part of his land, planting it tree by tree. His neighbors thought that he was crazy; four hectares of crops and forest were lost when arsonists started a blaze while Sawadogo was away for a morning. When people saw that he was successful, some came to learn his techniques. Slowly emigration stopped; some villages even saw their populations grow.

By 2009, Yacouba had about 12 hectares of forest and an extensive seed bank, with seeds divided by characteristics (such as fast growth and high yield). He and his farm had become valued resources for the community, and he continued to experiment with new techniques. Yacouba was invited to Washington, D.C., by Oxfam to talk about sustainable farming. Sadly, all of his achievements are at risk of being lost—not to desertification but to development. The city of Ouahigouya has taken the



land from him and his neighbors and divided it into small plots for multiple families. The acquisitions are part of an aggressive urban development plan, and Yacouba cannot afford the estimated €100,000 needed to buy back the rights to his holdings. So far, city officials have promised to leave the forest untouched, but there is already one house built next to it.

The Man Who Stopped the Desert alternates between a biography of Yacouba, beginning when he was sent to an Islamic school in Mali at age

7, and an homage to his work. Both narratives highlight his patience and determination in the face of adversity. He remains serene at the movie's end, despite the uncertainty surrounding the fates of his land and his livelihood.

—Trista Wagoner

Lagos/Koolhaas. Bregtje van der Haak, director. Pieter van Huystee Films, Netherlands. 2002. 55 minutes. www.pvhfilm.nl.



An unattended meadow can produce beautiful wildflowers, but weeds, trash, and pests may also accumulate. Is there a similar pattern of growth when a city is left unattended? Dutch architect Rem Koolhaas spent the late 1990s in Lagos, the capital of Nigeria, documenting the rapid urbanization resulting from explosive population growth. Large-scale infrastructure projects carried out in Lagos in the 1960s and 1970s provided a foundation for modernization and the absorption of increasing numbers of people. The sharp decrease in oil prices in the 1980s curtailed these efforts, leaving the increasingly crowded city awash in severe urban problems. Today, Lagos is on pace toward a 2020 population of 24 million, which would make it the third largest city in the world. How can a city with no organized planning and an incomplete infrastructure continue to function?

Carrying the audience deep into the chaos and commerce of Lagos, Koolhaas shows how inhabitants have learned to twist clear drawbacks to their advantage. For example, persistent traffic jams, produced by the permanent congestion, have evolved into hubs of entrepreneurial activity. Merchants place their stands at the very edge of the road, and buying and selling takes place through windows of stopped cars, turning inefficient roadways into efficient marketplaces. Incredible self-organization takes place in these autonomous entities—the most impressive of which, the Alaba market, started as a single stand on a narrow track road and now functions as the largest importer of electronics in West Africa. There each year, 50 thousand merchants conduct \$2.8 billion of trading, without the support of central governance or infrastructure. Instead, the Alaba market completely organizes itself and regulates its own law and order. (It even has a small prison run by the market owners.) Koolhaas uses such examples to illustrate Lagos's exceptional ability to function like an ecosystem and develop practically despite the absence of formal organization. He further suggests that Lagos may be redefining the idea of a city by showing that the Western notion of planning is an illusion. Perhaps all a city really needs to grow is a minimal framework that allows permanent and formal entities to mix with flexible and informal ones. In a state of astonishment, he concludes the film by commenting "Lagos is not catching up with us. Rather, we may be catching up with Lagos."

—Melissa McCartney

Carbon for Water. Evan Abramson and Carmen Elsa Lopez, directors. *Cows in the Field*, USA, 2011. 21 minutes. www.carbonforwaterfilm.com. Anzelma, age nine, lives in Western Province, Kenya. Each day, as Evan Abramson and Carmen Elsa Lopez's film *Carbon for Water* documents, she gets up before dawn to fetch the firewood needed to boil the water she and

her family will drink and cook with. As well as the loads she must carry on her head (bundles of logs, each as long as she is tall) and the punishment she might suffer from her parents if she refuses, she must also worry about being caught by the forest rangers—who, in her vivid imagination, might lock her away forever.

Boiling water is a necessity, as water-borne diseases (dysentery, typhoid fever, and cholera) are a very real threat where Anzelma lives. But having to boil water has three obvious drawbacks: It is an important contributor to deforestation. High concentrations of wood smoke in kitchen huts can lead to health complications. And the task represents a substantial time and energy sink for women and girls who must struggle for equality in this mostly traditional African society.

Boiling water has a fourth, less immediately obvious drawback: Burning wood releases carbon dioxide into the atmosphere, adding to the greenhouse effect that is driving global climate change. It is this last consequence that the European-based company Vestergaard Frandsen has leveraged in an attempt to ameliorate the problems surrounding the procurement of safe water for Anzelma to drink.

Over two months in 2011, the company delivered a simple-to-use water filtration system (gratis) to 900,000 homes in Western Province, with the intention of removing the need to boil water for drinking. A subsequent oral survey projects that these 900,000 “small interventions” will reduce CO₂ emissions by over 2 million metric tons per year. And that is how Vestergaard Frandsen will pay for the initial outlay and yearly maintenance of the filters: by selling the carbon they keep out of the atmosphere in the form of credits (one for every metric ton of CO₂) on the voluntary carbon market.

Anzelma (as serious as only a nine-year-old can be and our introduction to these rural communities) is irresistibly charming, the idea so simple, the aim so worthy, and the film so clearly lovingly made in its depiction of this



far-flung corner of Kenya that it is hard not to cheer along with CEO Mikkel Vestergaard Frandsen and his challenge at the end of the film: “Companies that don’t figure out and who don’t understand that they have this role [in maintaining environmental sustainability] probably won’t be around in ten or twenty years from now.”

Nonetheless, there are some sobering considerations: The project is slated to last for ten years, the time projected for the Kenyan government to pipe water into the region. If it does not, what then for the roughly 4.5 million people who currently benefit from the filter system? We are told the emissions reductions are rigorously monitored and audited, but what if the wood that might have been used for boiling is simply being burnt for another purpose? Similarly, will the time saved by the women and girls help empower them or merely shift the burden of their household workload? And, of course, Vestergaard Frandsen is also involved for the money. But if the project does work, wouldn’t it be worth the effort? Something to think about the next time you use perfectly drinkable water to flush the toilet.

—Guy Riddihough

Biophilic Design: The Architecture of Life. Bill Finnegan, director.

Tamarack Media, USA. 2011. 60 minutes. www.biophilicdesign.net.

When we think of “green” buildings, we usually think of sustainable design, with its emphasis on the conservation of energy, water, and materials. Bill Finnegan’s documentary, based on social ecologist Stephen Kellert’s book *Biophilic Cities*, explores the greening of buildings in a more literal sense. The biophilic design movement seeks to bring nature into the heart of buildings to provide a more harmonious habitat in which people can work and live.



The film opens with Kellert explaining that our cravings for contact with nature (biophilia) stem from the fact that, through evolution, our senses, emotions, and intelligence have been shaped by the natural world. Yet, modern buildings and cities tend to sever us from nature. Using interviews with several dozen architects and footage of exemplary buildings, the film describes elements of biophilic design and the benefits it brings.

Of course, the idea of recreating the natural world inside buildings is not new. It can be found in medieval cathedrals (with their use of natural materials, light, space, and animal and plant motifs) and in works by Frank Lloyd Wright (such as the residence Fallingwater, cantilevered over a waterfall in the highlands of Pennsylvania). Wright understood the importance of incorporating nature into designs for the workplace. In the film, as the camera zooms in on rows of gray, lifeless cubicles, an architect describes modern workplaces as “pretty tragic.” In contrast, lofty treelike columns in the great room of Wright’s Johnson Wax headquarters give workers an uplifting sense of savanna-like space.

The film takes us to recent office buildings and factory floors that have used biophilic design to boost productivity and workforce retention. These include a light-filled yurt that houses a cutlery factory (Sheffield, United Kingdom), the green-roofed YouTube headquarters (San Bruno, California), and the vast atrium of the Genzyme Center (Cambridge, Massachusetts). We also learn about health and medical benefits that accrue from biophilic design. Roger Ulrich discusses his classic 1984 experiment in which patients recovering from gall-bladder surgery who could view trees through their hospital window needed less pain medication, had fewer complications, and left the hospital sooner than their less fortunate counterparts who were left to gaze at a brick wall. Hospital architects took note, and designers now emphasize windows and natural light, views of trees and plants, and accessible gardens.

Finnegan’s film provides a pleasant overview of biophilic design. Although the approach contains more common sense than novelty, its application to homes, schools, office buildings, factories, and hospitals will help bring nature back into our lives—which can only do us good.

—Orla Smith

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Monitoring EU Emerging Infectious Disease Risk Due to Climate Change

Elisabet Lindgren,¹ Yvonne Andersson,² Jonathan E. Suk,³ Bertrand Sudre,³ Jan C. Semenza^{3*}

In recent years, we have seen transmission of traditionally “tropical” diseases in continental Europe: chikungunya fever (CF) in Italy in 2007, large outbreaks of West Nile fever in Greece and Romania in 2010, and the first local transmission of dengue fever in France and Croatia in 2010 (1–3). These events support the notion that Europe is a potential “hot spot” for emerging and re-emerging infectious diseases (EIDs) (4). Major EID drivers that could threaten control efforts in Europe include globalization and environmental change (including climate change, travel, migration, and global trade); social and demographic drivers (including population aging, social inequality, and life-styles); and public health system drivers (including antimicrobial resistance, health care capacity, animal health, and food safety) (5, 6). Climate change is expected to aggravate existing local vulnerabilities by interacting with a complex web of these drivers (6). For example, increases in global trade and travel, in combination with climate change, are foreseen to facilitate the arrival, establishment, and dispersal of new pathogens, disease vectors, and reservoir species.

Complexity is not an excuse for inaction. An effective public health response to climate change hinges on surveillance of endemic and emerging diseases (7). Infectious disease surveillance at the European level is regulated by the European Parliament and Council (8). In 2009, the European Commission outlined actions needed to strengthen the European Union’s (EU) resilience to the impacts of changing climate (9), specifically on the surveillance of health effects such as infectious diseases. The European Centre for Disease Prevention and Control (ECDC) was charged with guiding this process, which is summarized below. It included extensive literature reviews, eval-

uation of current surveillance systems in Europe, weighted risk analysis of different diseases, and expert consultation with EU member state representatives (10, 11).

Novel Approaches to Risk Assessment and Surveillance Are Needed

Currently, risk analyses tend to focus on identifying diseases that are climate-sensitive. Evidence-based risk assessments with clearly defined uncertainties and underlying assumptions should be routinely undertaken to identify disease risks from climate change and to prioritize possible changes in surveillance. However, a comprehensive climate change assessment needs to be expanded to account for societal aspects so as to fully reflect the impact of climate change. The results from such combined, weighted analyses can be used to evaluate whether surveillance ought to be implemented or modified.

Current EU-wide surveillance is either indicator-based (annual country-level reporting of confirmed human cases) or event-based (detection of individual disease outbreaks through epidemic intelligence). Traditional surveillance often does not suffice for early detection of EIDs. EU member states currently report confirmed human cases of notifiable diseases to ECDC, animal cases of zoonotic diseases to other EU registries, and food-borne and zoonotic outbreaks to the European Food Safety Authority. Reporting of bathing and drinking water quality data are governed by other EU directives, and vector surveillance is voluntary. Early detection of EIDs related to climate change calls for fine-tuning of current surveillance approaches; for example, increased cross-sectoral interactions and sentinel surveillance are warranted.

Climate Change and Infectious Diseases

Notable changes in annual average temperature and mean precipitation are predicted for Europe, with disproportionately warmer winters in the north and warmer summers in the south (12). The impacts of climate change on infectious diseases are complex and multifaceted (11, 13) (see table S1 in the supplementary materials). Changes in precipitation amounts and patterns can bring

Climate change, globalization, and other drivers have made Europe a “hot spot” for emerging infectious diseases, which calls for changes in monitoring systems.

about increases in water flows and floods, leading to contamination of drinking, recreational, or irrigation water and increased risk of outbreaks of cryptosporidiosis and vero toxin-producing *Escherichia coli* (VTEC) infections, for example. Higher water temperatures increase the growth rate of certain pathogens, such as *Vibrio* species that can cause food-borne outbreaks (seafood) or, on rare occasions, lead to severe necrotic ulcers, septicemia, and death in susceptible persons with wounds bathing in contaminated waters. Elevated air temperatures could negatively affect the quality of foodstuff during transport, storage, and food handling. Increasing temperatures typically shorten arthropod life cycles and the extrinsic incubation periods of vector-borne pathogens, potentially leading to larger vector populations and enhanced transmission risks. Long-term seasonal changes will affect both vectors and host animals and may locally affect land-use changes and human behavior, with implications for the geographical distribution, seasonal activity, and prevalence of many vector-borne diseases in Europe (11).

Weighted Risk Analysis

EU member states are currently required to report 46 infectious diseases and 7 additional diseases if they cause hemorrhagic symptoms. In addition, nosocomial infections (i.e., hospital-acquired) and antimicrobial resistance are also mandatory to report. Of the total reportable diseases, 26 were found to be either directly or indirectly affected by climate change, on the basis of a systematic literature review and expert judgment (see the supplementary materials). Of the nonreportable diseases, five climate-sensitive diseases were considered to be EIDs due to climate change (10, 11) (see table S1 in the supplementary materials).

Based on the literature and expert judgments (10, 11), the strength of association with climate change in Europe was categorized as low, medium, or high. Yet, even a strong link with climate change did not necessarily imply the need for surveillance; prevalence, severity, and secondary complications (including human and financial

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Strength of link with climate change in Europe					Weighted high risk	
	Low		Medium			
	High		High			
	Low		Medium			
High			<i>Vibrio</i> spp. (except <i>V. cholerae</i> O1 and O139)* Visceral leishmaniasis*		Lyme borreliosis*	
Medium	CCHF Hepatitis A Leptospirosis	Tularaemia Yellow fever Yersiniosis	Campylobacteriosis Chikungunya fever* Cryptosporidiosis Giardiasis Hantavirus	Rift Valley fever Salmonellosis Shigellosis VTEC West Nile fever	Dengue fever TBE*	Weighted medium risk
Low	Anthrax Botulism Listeriosis Malaria	Q fever Tetanus Toxoplasmosis	Cholera (O1 and O139) Legionellosis Meningococcal infection			Weighted low risk
Potential severity of consequence to society						

Weighted risk analysis of climate change impacts on infectious disease risks in Europe. CCHF, Crimean-Congo hemorrhagic fever. Candidates for suggested changes to disease-specific surveillance are in bold. Asterisks indicate diseases currently notifiable in some EU member states but not legally reportable to ECDC.

costs for society) were important considerations in this weighted analysis. By combining these parameters, the importance of disease for society was ranked as low, medium, or high. These values were then plotted on a matrix (see the figure) to reflect the joint importance of climate change and severity of a specific disease in Europe.

Several of the climate-sensitive reportable diseases are found in the medium category. Most of these are already under adequate surveillance, even if climate change further increases disease risk, but seven could justify changes in the surveillance system (see the figure). Hemorrhagic cases of dengue fever (DF) and Rift Valley fever (RVF) are currently reported to ECDC but are all imported cases. However, increased risk of autochthonous (indigenous, not imported) transmission demands heightened surveillance.

Surveillance of EID Threats from Climate Change

The recent emergence of DF and CF in Europe has been traced to environmental conditions conducive to high vector population densities of the invasive Asian tiger mosquito, *Aedes albopictus*, and permissive temperatures for pathogen replication within the vector (1). Surveillance of DF and CF will require a combination of vector surveillance—to detect new risk areas and human case reporting—with a focus on the warmer parts of the year to detect autochthonous cases.

Monitoring established vectors close to their altitude and latitude distribution limits will help to delineate expanding and contracting areas of risk. Human case reporting of Lyme borreliosis could potentially be linked to sentinel monitoring of infected

ticks in selected areas, a practice already implemented in the Czech Republic and planned for Denmark. Using the disease-specific skin rash, erythema migrans, as the case definition of Lyme borreliosis would pick up shifting distribution and risks, rather than the rare laboratory-confirmed Lyme neuroborreliosis. For the detection of possible new risk areas of leishmaniasis, surveillance of the sandfly vectors could be coupled with monitoring of infected pet dogs in the EU. Tick-borne encephalitis (TBE) is best surveyed through reporting of serologically confirmed human cases.

Establishing surveillance for the introduction of new vector species into the EU could contribute substantially to infectious disease control, particularly when linked with surveillance of imported human and/or animal cases. Enhanced collaboration between the veterinary surveillance and public health sector will advance preparedness and response if pathogens and vectors become prevalent in the region and pose a threat to humans. Lessons learned from pandemic avian flu preparedness could be applied to other EIDs. Collaboration between the human and veterinary sectors was considerably improved through such practices as regular meetings, routine sharing of epidemiologic and laboratory data, preparation of linked response plans for human and veterinary health, and coordinated outbreak investigations.

Important food-borne and waterborne diseases, in particular the zoonoses, are already part of surveillance collaborations in Europe. However, new collaborations will be needed. Surveillance of *Vibrio* spp. outbreaks due to seafood consumption could be enhanced by initiating collaborations between human case

surveillance and laboratory monitoring of bivalve shellfish contamination.

These challenges call for the development of improved surveillance by monitoring environmental precursors of disease. ECDC is developing the European Environment and Epidemiology (E3) Network, designed to link environmental with epidemiological data for early detection of and rapid response to shifting infectious disease burden (11). ECDC is also developing VBORNET, a surveillance tool used for monitoring the distribution of invasive disease vectors within the EU.

Conclusions

Adjustments to existing surveillance practices in the EU, as outlined above, will enhance preparedness and facilitate the public health response to EIDs and thereby help contain human and economic costs (14). These benefits need to be balanced against the additional costs of program implementation; however, cost-benefit analyses tend to be hampered by the inherent difficulty in attributing EID impacts to specific climate change events. Nevertheless, these policy-driven adaptation measures should facilitate early detection of EIDs, regardless of the underlying macro-level drivers.

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Supplementary Materials

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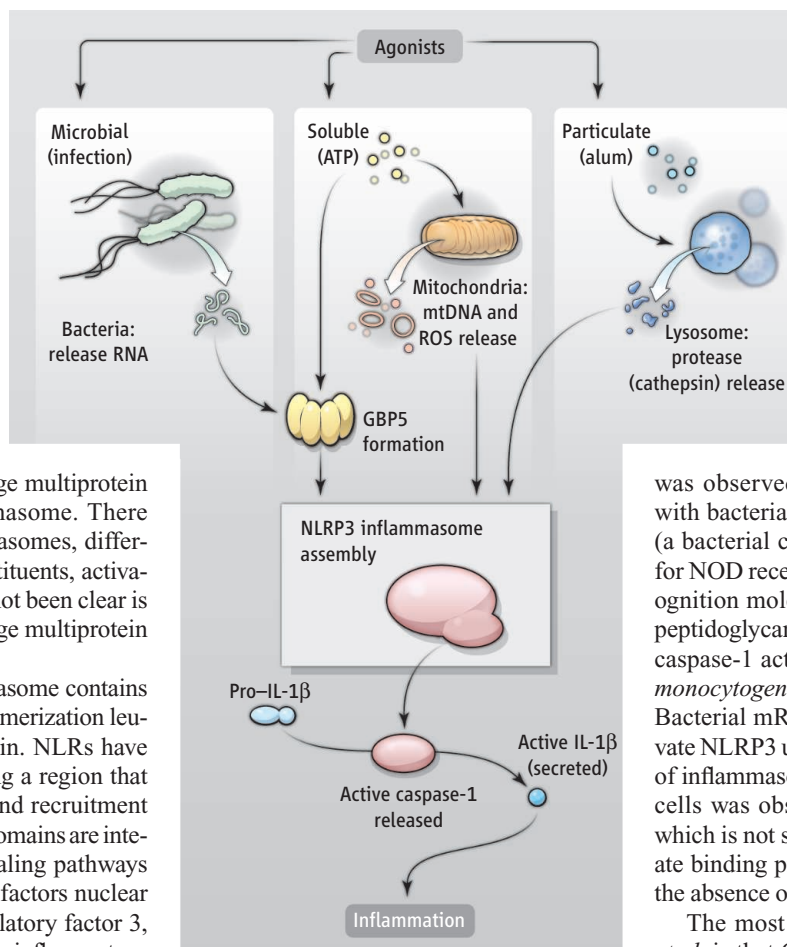
Select Inflammasome Assembly

Daniel R. Caffrey and Katherine A. Fitzgerald

Some pairs of interacting proteins have homologous domains in other species that are fused together to form a single multidomain protein. This observation has been successfully exploited by various computational methods that predict interactions between different protein domains (1, 2). On page 481 in this issue, Shenoy *et al.* (3) use a similar approach to identify a protein in macrophages that selectively induces the assembly of a large multiprotein complex called the inflammasome. There are distinct types of inflammasomes, differentiated by their protein constituents, activators, and effectors. What has not been clear is how the assembly of these large multiprotein structures is controlled.

In most cases, an inflammasome contains a nucleotide binding and oligomerization leucine-rich repeat (NLR) protein. NLRs have a tripartite structure, including a region that can be a caspase activation and recruitment domain (CARD) (4). CARD domains are integral components of cell signaling pathways that activate the transcription factors nuclear factor κ B and interferon regulatory factor 3, as well as proapoptotic and proinflammatory proteases (caspases). In response to diverse microbial, environmental, or endogenous danger signals, inflammasome complexes assemble to recruit and activate caspase-1 (see the figure). Whereas some NLR proteins recruit caspase-1 directly, others like NLRP3 require an adaptor molecule to do so. Recruitment of caspase-1 leads to caspase-1 autoactivation, which in turn cleaves interleukin-1 β (IL-1 β) and IL-18 into mature biologically active cytokines. Upon release from immune cells, these cytokines have wide-ranging acute and chronic inflammatory effects.

The guanylate binding proteins are part of a larger family of interferon- γ (IFN- γ)-inducible cytoplasmic guanosine 5'-triphos-



Inflammasome assembly. Several mechanisms have been proposed by which microbial, endogenous, and particulate agonists activate the NLRP3 inflammasome. GBP5 is involved in assembling the inflammasome in response to soluble and microbial agonists. ROS, reactive oxygen species.

phatases (GTPases) in mammalian cells, several of which have been linked to antimicrobial defenses (5). Two guanylate binding proteins in zebrafish (GBP3 and GBP4) contain a GTPase domain fused to a CARD domain (3). Based on the assumption that these two domains interact within a single protein chain, Shenoy *et al.* reasoned that human guanylate binding proteins might bind to other proteins that contain one or more CARD domains. Using small interfering RNAs corresponding to the complete human and mouse GBP families, Shenoy *et al.* explored the potential interaction of gua-

Inflammasomes that respond to pathogenic bacteria assemble in response to a specific cytoplasmic protein in macrophages.

nylate binding proteins with NLR inflammasome complexes. Of the 17 GBP family members tested, human GBP5 and its mouse ortholog were uniquely required for NLRP3-dependent IL-1 β secretion. Studies in both human monocytes, as well as in macrophages from GBP5-deficient mice, identified GBP5 as a key mediator of caspase-1-dependent IL-1 β secretion elicited by endogenous adenosine 5'-triphosphate (ATP). This was observed when cells were stimulated with bacterial lipopolysaccharide, nigericin (a bacterial compound), or specific ligands for NOD receptors (intracellular pattern-recognition molecules that recognize bacterial peptidoglycan). GBP5 was also required for caspase-1 activation in response to *Listeria monocytogenes* or *Salmonella typhimurium*. Bacterial mRNA from both pathogens activate NLRP3 upon infection (6). Notably, loss of inflammasome activity in GBP5-deficient cells was observed in IFN- γ -treated cells, which is not surprising given that all guanylate binding proteins are poorly expressed in the absence of IFN- γ treatment (5).

The most surprising finding of Shenoy *et al.* is that GBP5 is selectively required to activate the NLRP3 inflammasome by soluble agonists such as ATP, but dispensable for NLRP3 activation by crystalline compounds [alum and monosodium urate (MSU)]. Although these particulate agonists lead to assembly of the NLRP3 inflammasome, they appear to do so through a different mechanism. Destabilization of the phagolysosomal compartment after engulfment of alum and MSU and subsequent release of lysosomal cathepsins are important for activating the NLRP3 inflammasome (7). However, the precise molecular mechanisms by which soluble, particulate, or microbial (bacterial messenger RNA) agonists signal the assembly and activation of the NLRP3 inflammasome are still unclear. None of these ligands bind or act as direct agonists of NLRP3. Several theories have been proposed, none of which are mutually exclusive, to explain these events. Potassium efflux, the release of cathepsins

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from damaged lysosomes, the generation of reactive oxygen species, and the release of DNA from damaged mitochondria have all been linked to NLRP3 activation (8–10).

By carefully ruling out potential upstream mechanisms, Shenoy *et al.* suggest that GBP5 acts at a level proximal to the caspase-1 constituent of the inflammasome. GBP5, but not GBP1 (a related paralog), binds to the NLRP3-pyrin domain and thereby promotes oligomerization of NLRP3. The authors generated mutant forms of GBP5 [by modeling GBP5 to the known structure of GBP1 (11)] and found mutants that favored the formation of a tetrameric GBP5 complex. They propose that these “activated tetramers of GBP5” subsequently stimulate NLRP3 inflammasome assembly. A regulatory switch mechanism likely exists to convert GBP5 to this tetrameric form to promote NLRP3 complex assembly.

Why is GBP5 required for some but not all NLRP3 agonists to promote inflammasome assembly? Perhaps other GBP family members promote the assembly of NLRP3 in

response to particulate ligands or aid in the assembly of other NLRs. Another question is how the GBP5 “switch” is activated. ATP and nigericin cause the oxidation and release of DNA from mitochondria (mtDNA) into the cytoplasm (9, 10). Binding of oxidized DNA to NLRP3 then promotes assembly and oligomerization of the NLRP3 inflammasome (10). Future studies should determine if and how the work of Shenoy *et al.* fits with this model.

GBP5-dependent assembly of the NLRP3 inflammasome likely occurs in situations where IFN- γ is present. Whether GBP5 facilitates the initial wave of NLRP3 activation or acts to amplify these events when IFN- γ is released from natural killer and T cells at sites of inflammation or infection remains to be clarified. Shenoy *et al.* identify GBP5 as a positive regulator of IFN- γ -dependent inflammasome signaling, whereas IFN- α/β and IFN- γ can apparently restrain NLRP3-driven inflammation by antagonizing pro-IL-1 β production and caspase-1 activation (12, 13). Whether IFN- γ exerts posi-

tive GBP5-dependent or negative regulatory effects likely depends on the cellular context. Regardless, the identification of GBP5 raises the intriguing possibility that mutations in GBP5 could contribute to human inflammatory and infectious diseases linked to dysregulation of the NLRP3 inflammasome.

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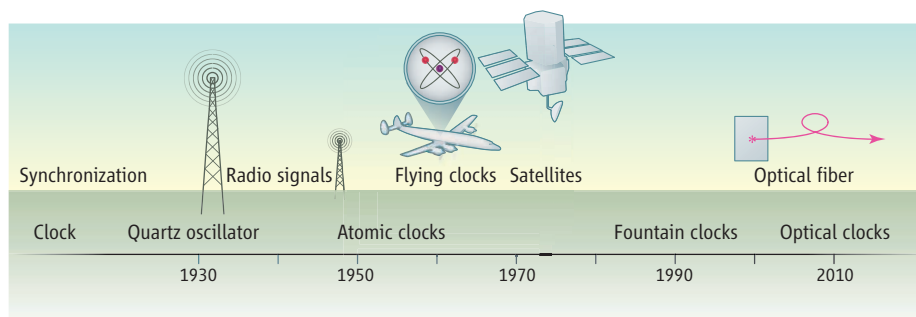
PHYSICS

Two Atomic Clocks Ticking as One

Bruce Warrington

How do you set your watch? Perhaps you set it from a radio broadcast, or your computer, or your mobile phone. Each of these devices gets its time in turn from a hierarchy of clocks, typically terminating at a national institute that keeps the reference standards of measurement in your country. If your watch is stable, how well you can rely on it depends on how well you can set it, and thus on propagation delays and “jitter” (phase noise) in the propagation of the reference timing signal itself—whether by radio or over a computer or telecommunication network. In practice, your wristwatch is not stable enough for this to matter, but it is a problem for the best atomic clocks in the national institutes. These are now so stable that they cannot be compared by any of these means, or even by satellite, without noise in the comparison swamping the stability of the clocks. Without new techniques, such as long-range transfer over optical fiber reported on page 441 of this issue by Pre-

Optical fiber networks allow synchronization of distant optical clocks, which tick too fast to be linked via satellite signals.



A timeline of timekeeping. Two problems in timekeeping—improving the accuracy of measuring time and synchronizing time pieces—are intimately related. The timeline illustrates some of the developments in both of these fields.

dehl *et al.* (1), we will not be able to take full advantage of these clocks in international timekeeping, and the amazing progress of atomic clocks may grind to a halt.

This problem is not new. Historically, the ability to keep time with a single clock has always been separate from the ability to compare two distant clocks, with technical advances leapfrogging each in turn into the lead (see the figure). In the mid-20th century, the advent of atomic clocks separated time from the rotation of Earth; a new definition

of the second was determined by comparing atomic time with astronomical time (2), linking laboratories in the United Kingdom and the United States by radio transmission. The new clocks became more and more stable, until radio could no longer transfer time signals without degrading their stability.

The only solution was to bring the clocks physically together for comparison. Elaborate “flying clock” campaigns circulated a portable atomic clock by air (3) for international comparisons of clocks in national

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institutes (4). The advent of satellite techniques (5) restored the ability to compare clocks remotely without degradation, and the balance remained essentially this way through the remainder of the 20th century. This ability enabled first the coordination of national time signals (6) and eventually the generation of International Atomic Time (TAI) and Coordinated Universal Time (UTC). These time scales are now the basis of international timekeeping, drawing on and linking together hundreds of atomic clocks around the world (7) and in turn supporting global applications from electricity distribution to financial markets.

Today, the best atomic clocks tick at optical frequencies ($\sim 10^{15}$ Hz), and are orders of magnitude more stable again than their microwave forebears ($\sim 10^{10}$ Hz). Two optical clocks in the same laboratory can be compared (8) with exquisite precision. General relativity predicts that if a clock on Earth is elevated, it runs faster (because of its higher gravitational potential), and this effect has been resolved for a change in height of only 33 cm or a fractional frequency shift at the 10^{-17} level (9). However, for distant clocks, even the best satellite comparisons stop well short of this resolution, by a factor of ~ 100 after 1 day of averaging (10). Without new techniques, we must return to the flying clock comparisons of 50 years ago, and await the development of a portable optical frequency standard, an undertaking being pursued in several laboratories.

Transfer of signals by optical fiber is one possible solution. A number of links have been successfully demonstrated for distances of around 100 km, and, with active stabilization of fluctuations in length, can transfer the full performance of optical standards within practical averaging times. The goal is now to reach longer distances: 1000 km or more to link laboratories around Europe, several thousand kilometers to span the United States or Australia, and even longer for intercontinental links. The work of Predehl *et al.* sets a new distance record (920 km), but more importantly, it shows that the practical challenges such as attenuation and remote operation can be met on this distance scale. Separately, it has also been shown that these comparisons can be performed in parallel with data transfer over the same “lit” fiber—that is, one already in use on other wavelength channels (11). This capability is important, as it extends the reach of networks where “dark” fiber (installed in anticipation of future capacity increases) proves impractical or prohibitively expensive.

Together, these technologies are working toward a new international network of time and frequency standards. This network is not just for metrologists, but supports a whole range of applications, just as did earlier networks linked by telegraph, radio, or satellite. One example is precision geodesy, measuring Earth’s gravitational potential to new levels of precision with optical clocks as “Einstein sensors.” Precise syn-

chronization is also needed for very long baseline interferometry in radio astronomy, with antenna systems such as the proposed Square Kilometre Array extending over continental scales. Here the same fiber network that transfers data can also transfer time; in future, the same can be true for research, industry, and even our homes, with unprecedented access to accurate and reliable time.

In the 60 years or so since the development of the atomic clock, technological infrastructure from telecommunications networks to satellite navigation systems has come to depend intimately not only on the performance of these clocks but also on the ability to compare them. This history tells us to expect the revolution to continue during the next 50 years of timekeeping.

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EVOLUTION

Microbial Evolution in the Wild

Edward F. DeLong

Microbial species thrive in every corner of Earth’s biosphere. Their rapid growth rates and promiscuous gene swapping provide ample grist for the evolutionary mill over relatively short time spans. In ocean surface waters, for example, microbial doubling times of about 1 day result in an estimated production of around 10^{30} new cells per year (1). (This number exceeds that of all individual grains of sand on Earth by about 10 orders of magnitude.) These cells are not static entities, but rather the products and perpetuators of dynamic

evolutionary processes. But exactly how fast, to what degree, and by what mechanisms, do free-living microbes change and evolve over time in natural settings? On page 462 of this issue, Denef and Banfield (2) report evolutionary rate estimates from free-living microbial species in the wild.

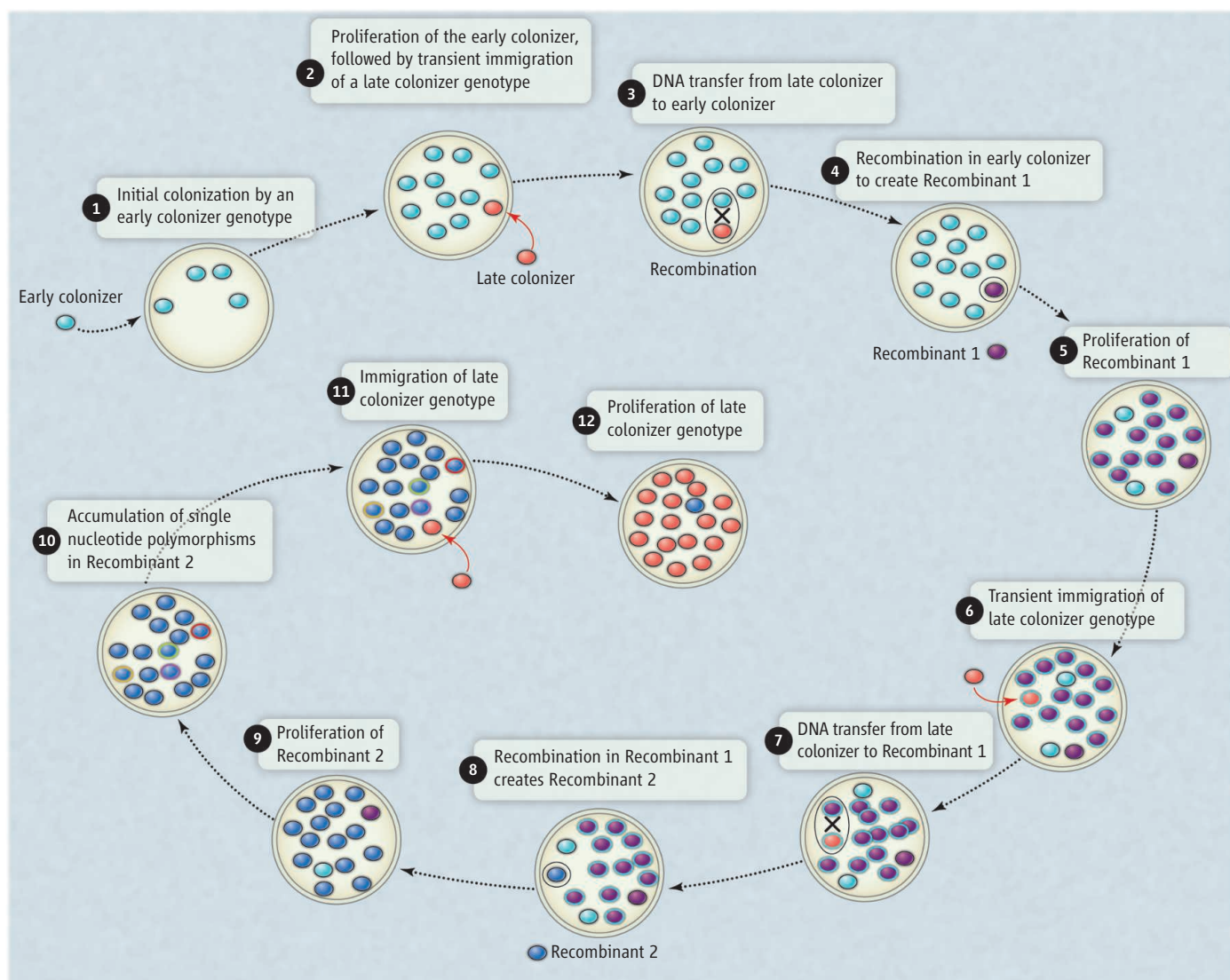
Measuring evolutionary rates of individual species in complex natural microbial assemblages is a daunting task. Yet Denef and Banfield faced even more formidable challenges. The hot, humid, acidic, low-oxygen conditions found at their acid mine drainage (AMD) study site is, as the authors put it, “close to the limit of human endurance” (2). This would seem an unlikely place for conducting detailed evolutionary studies—but in

A genetic study of microbes in an acid mine drainage site provide evolutionary rate estimates for wild microbe populations.

some ways it is ideal. The AMD site harbors a very simple ecosystem containing only a handful of microbial species (3). *Leptospirillum*, a hardy and versatile bacterium that can live in sulfuric acid, eat iron, and fix carbon dioxide, often dominates AMD biofilms (3, 4). The very low diversity and complexity of the AMD biofilm allowed Denef and Banfield to directly follow the succession of genotypes in *Leptospirillum* populations over time, and to track recombination events and the accumulation of genomic nucleotide substitutions in populations collected over the course of 5 years (see the figure).

By comparing assembled genomes from different genotypes against total population DNA, the authors found that the AMD bio-

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film populations contained six distinct *Leptospirillum* genotypes (types I to VI). The time series was dominated by *Leptospirillum* genotype III, which appeared to be a recombinant hybrid of genotypes I and VI. Time-series population DNA sequencing of *Leptospirillum* genotype III biofilms sampled over the course of 5 years yielded a single-nucleotide substitution rate of 1.4×10^{-9} substitutions per nucleotide per generation. This rate estimate, along with evolutionary history reconstructions, suggested that the six *Leptospirillum* genotypes diverged from a common ancestor in a matter of decades. The data also indicated that the major recombinations reflected single events that led to the domination of specific recombinant genotypes. Population genomic analyses suggested that *Leptospirillum* genotype evolution (see the figure) consisted of a complex menu of immigration, horizontal gene transfer (HGT), homologous recom-

ination, fixation, and selective sweeps that successively generated and replaced different genotypes and, presumably, phenotypes.

The AMD *Leptospirillum* nucleotide substitution rate of 1.4×10^{-9} per nucleotide per generation is at the high end of previous estimates, which have ranged from 7.2×10^{-11} to 4.0×10^{-9} (5–7). One reason may be the use of a different approach. In perhaps the most elegant and comprehensive study of its kind to date, Denef and Banfield performed cultivation-independent population genomic analyses. In addition, they leveraged a unique combination of data that included in situ proteomic data sets (8), three distinct *Leptospirillum* population genotypes (assembled directly from natural populations) and a population genomic time series.

Unlike Denef and Banfield's study, other estimates of bacterial evolutionary rates have relied on isolation, propagation, or

Microbial microevolution in a biofilm. This hypothetical scenario of evolutionary events in AMD biofilms illustrates some of the processes observed and inferred by Denef and Banfield. The white circles do not necessarily represent the same biofilm. The dotted black arrows do not imply direct succession in the same biofilm; rather, different amounts of time, and different spatial locations, may be involved in the transitions, which could take years to decades.

comparison of pure cultures or laboratory populations (5, 7, 9). These approaches may be selective and may not reflect native community complexity. On the other hand, the AMD habitat is itself unique; its unusual conditions and low biological complexity may affect the evolutionary rate estimates. Denef and Banfield point out, however, that their estimates are consistent with independent theoretical predictions of a universal mutation rate (10).

A central goal in microbial population biology is to identify key genomic changes

that lead to adaptation and selection—the “differences that make a difference” (11–13). Denef and Banfield speculate that phenotypic selection in the *Leptospirillum* populations may be due to differences of only a few nucleotides, but there are some caveats. Their approach captured major homologous recombination events, but nonhomologous gene acquisitions from HGT may have gone undetected, because the *Leptospirillum* population analyses relied on comparisons of genes present in reference genomes. Without complete and contiguous genome assemblies from the population genomes, large blocks of newly acquired foreign genes could be invisible to this approach.

Recent studies suggest that HGT and illegitimate recombination can be common and frequent in native microbial populations (13–15).

It turns out that “genome” is a verb, not a noun—a process, not a product. Current and emerging microbial population genomic studies that capture this dynamic (2, 6, 7, 9, 13–15) promise to help paint a more integrated motion picture of microbial ecology and evolution in action.

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ASTRONOMY

Complex Protostellar Chemistry

Joseph A. Nuth III and Natasha M. Johnson

Two decades ago, our understanding of the chemistry in protostars was simple—matter either fell into the central star or was trapped in planetary-scale objects. Some minor chemical changes might occur as the dust and gas fell inward, but such effects were overwhelmed by the much larger-scale processes that occurred even in bodies as small as asteroids. The chemistry that did occur in the nebula was relatively easy to model because the fall from the cold molecular cloud into the growing star was a one-way trip down a well-known temperature-pressure gradient; the only free variable was time. However, just over 10 years ago it was suggested that some material could be processed in the inner nebula, flow outward, and become incorporated into comets (1, 2). This outward flow was confirmed when the Stardust mission returned crystalline mineral fragments (3) from Comet Wild 2 that must have been processed close to the Sun before they were incorporated into the comet. On page 452 of this issue, Ciesla and Sandford (4) demonstrate that even the outermost regions of the solar nebula can be a chemically active environment. Their finding could have consequences for the rest of the nebula.

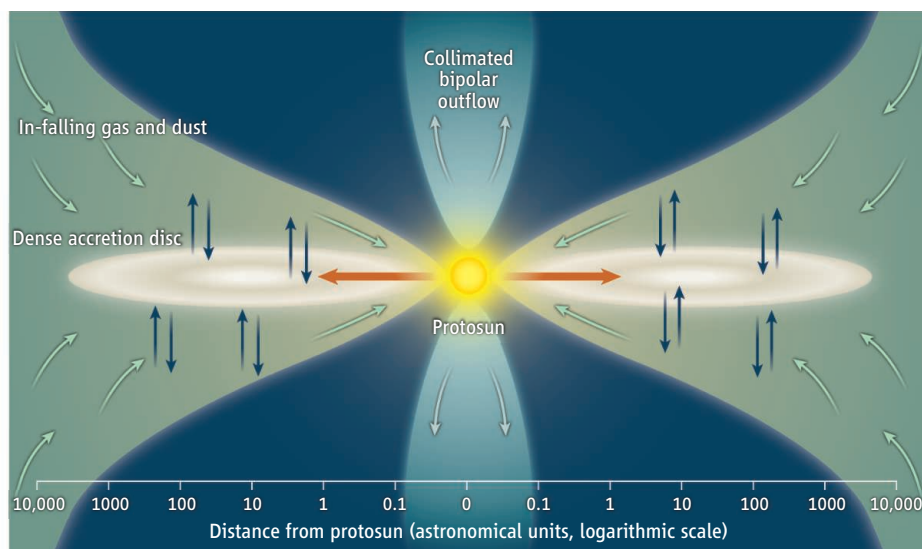
Outward flow in the nebula is the natural result of conserving the total angular momentum of the star-forming system as mass continues to accrete onto the rotating disk feed-

ing the growth of the central star (5–7). As mass flows inward along the flared accretion disk, additional mass flows slowly outward along the midplane (see the figure). As an additional complication, because the midplane is hotter than the outer boundary of the disk, convection will also mix materials vertically in the nebula. Ciesla and Sandford show that ice-coated dust grains, moving outward and subject to convection, will be exposed to cosmic radiation that is sufficient to cause the same chemical effects seen in dark cloud cores—that is, the conversion of simple car-

Numerical models show that protoplanetary nebulae are sites of chemical activity even in the cold outer disk.

bon- and nitrogen-containing molecules into more complex organic species—and so will have consequences for nebular chemistry.

It has been nearly 40 years since the discovery that the oxygen in solid bodies throughout the solar system is mass-independently fractionated (8). It appears as if ^{16}O were added or subtracted from the bulk composition of the dust independently of either ^{17}O or ^{18}O . While the first explanation for this observation was the addition of ^{16}O -rich supernova grains to the forming solar system (8), the currently favored explanation is known as chemical



Complex reactions. Large-scale motion driven by conservation of angular momentum, together with more local convective cells above and below the hotter nebular midplane, dynamically mix products of chemical reactions from many different environments throughout the nebula.

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self-shielding (9–11). The principle is similar to that of laser isotopic separation of gases, but uses either interstellar ultraviolet (UV) radiation or the light of the growing Sun. In either case, UV radiation dissociates CO molecules. However, owing to the abundance of $C^{16}O$ molecules, the radiation penetrating the system runs out of photons capable of breaking up this isotopic combination before it runs out of photons that dissociate $C^{17}O$ or $C^{18}O$. The result is that in such regions, ^{17}O and ^{18}O are free to react, either directly with dust grains or with H atoms to form H_2O that eventually reacts with the dust. Meanwhile, the extremely stable $C^{16}O$ sequesters the ^{16}O in the gas phase and prevents it from reacting with silicates. As long as CO remains stable, the self-shielding mechanism remains a viable explanation for the mass-independent fractionation of oxygen isotopes throughout the solar system.

Unfortunately, Ciesla and Sandford demonstrate that CO is not a stable sink for ^{16}O , and that such molecules will be converted into complex organics even in the cold outer reaches of the solar nebula, just as they are in cold molecular cloud cores. If CO is converted into organic molecules by radiation processing in icy grains in the outer nebula, just as it

is via Fischer-Tropsch-type reactions (12–14) on the surfaces of warmer grains in the inner nebula, and if such organics are free to react with silicate dust or to react with the bulk silicate in planetesimals, then the effects of photochemical self-shielding will be minimized or even completely erased. A new mechanism may be needed to explain the oxygen isotopic fractionation observed in the solid bodies of the solar system.

Our understanding of the chemistry in protostellar systems has made enormous progress over the last few decades, fueled by an increased awareness of the complex dynamics of these evolving energetic nebulae. We can no longer consider just the simple local environment (15) to explain the composition of a planet, asteroid, or comet as was done in the past, but must now consider chemical processes that might take place within the nebula as a whole as well as the probability of transport and mixing the products of such reactions throughout the system. Just as we now find it impossible to explain the complex chemistry of the terrestrial atmosphere without reference to detailed transport models that interconnect highly dissimilar chemical environments, so chemical models of protostars and of the

solar nebula must eventually treat these environments as tightly coupled, interactive systems. The demonstration that the chemistry on the surfaces of outward-flowing, dynamically mixing icy grain surfaces both mimics the chemistry in cold cloud cores and strikes at the central assumption of the photochemical self-shielding model for oxygen isotopes in solar system solids only adds emphasis to this conclusion.

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CELL BIOLOGY

Using Cell-to-Cell Variability— A New Era in Molecular Biology

Lucas Pelkmans

Every cell biologist who has used a microscope knows that single cells in a population display variable behavior (1). Although heterogeneity between single cells is obvious in tissues and organisms, it can also be observed in populations of monoclonal cells that have been cultured under identical conditions. Besides having stochastic sources (2, 3), phenotypic cell-to-cell variability among genetically identical cells can be deterministic and regulated, in both prokaryotic and mammalian cells (4, 5). Molecular and cell biologists have traditionally ignored this phenomenon, in part because of technical limitations, but also because, historically, research has focused on mechanisms and processes that are common between cells.

However, the mechanisms that make them different are likely to be equally important. Embracing this cell-to-cell variability as a fact in our scientific understanding requires a paradigm shift, but it will be necessary. In fact, it may be a strong boost for the field by uncovering novel and previously overlooked mechanisms of regulation at the cell population level.

Uncovering a novel mechanism, structure, or organelle that is generally observed in all cells is still regarded as the ultimate goal for most scientists in the field. Although clearly important, this fixation on results that can be generalized while dismissing other, conflicting findings has hindered progress. Fortunately, the field is slowly accepting that multiple mechanisms often exist for a particular cellular behavior or activity and that these mechanisms can vary between individual cells, even within the same monoclonal population (6–8).

Studying the phenotypic differences between genetically identical cells rather than their general features can reveal novel regulatory mechanisms for diverse cellular processes.

The great value of cell-to-cell variability for uncovering novel cellular processes comes from its being a natural source of “perturbation.” A similar approach is applied in the social sciences and economics, where relationships between individual factors are inferred through the analysis of variability in these factors in dynamically changing social and economic systems (9). However, these sciences can generally only make use of naturally occurring perturbations to test hypotheses. In contrast, the tools available to the molecular cell biologist provide some unique advantages, such as combining the analysis of cell-to-cell variability with intentional, targeted molecular perturbations. In addition, with the advent of high-throughput image-based technologies and computational image analysis (10, 11), large data sets can be constructed comprising measurements from multiple molecular and phenotypic states in large numbers of single cells within their

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Above average. Single, genetically identical (monoclonal) cells (circle, half circle, pentagon, and square) are not the same as the average cell (triangle). Individual cells may display many cell-to-cell variabilities, such as in the signaling kinases mitogen-activated protein kinase (MAPK: half circle) and mammalian target of rapamycin (mTOR: pentagon), and in clathrin-mediated (circle) or caveola-mediated (square) mechanisms of endocytosis, making each individual cell clearly different.

microenvironment over long periods of time. These data sets are particularly well suited for statistical approaches to find physiologically relevant connections, such as causality and feedback (12). Once such approaches become mainstream, we may find that cell-to-cell variability is fundamental to most, if not all, molecular cellular processes, from the number of ribosomes in a cell or its metabolic capacity to cytoskeletal properties and the activity of signaling pathways. One consequence of this will be the realization that the concept of a single “textbook-model” cell does not factually exist (see the figure).

We all struggle with the amount and the complexity of molecular information available on the regulation of cellular processes, as well as the layers of cross talk between them. Most of this knowledge has been obtained with cell population-averaging techniques or

from a relatively few single cells and then generalized to all cells. However, it is likely that when these processes are studied at the single-cell level, it becomes clear that not all parts of a complex pathway (e.g., of signal transduction) or all existing alternative routes (e.g., of endocytosis) act together in an individual cell, but rather in different subsets of cells within one population. Thus, we may find that this molecular complexity manifests itself in part at the cell population level and can be explained by studying patterns of cell-to-cell variability.

A rich hunting ground for molecular cell biology is to find out the molecular mechanisms by which regulated cell-to-cell variability emerges in a population. Quantitative measurements of how cells spatially organize themselves in a growing population will reveal how heterogeneity in the single-cell microenvironment is created (5). Integrating this with molecular measurements of important signal transducers will explain how cell-to-cell variability patterns of cellular signaling emerge. This, in turn, may affect a wide range of cellular processes. For instance, it may cause heterogeneity in transcription factor activity and subsequently in gene transcription (13, 14), and it may affect signaling pathways that control protein translation (15) or metabolic rate (16). At a higher

level, this may cause variation in the state of the cytoskeleton, lipid composition of membranes, and membrane trafficking (5), as well as in intrinsically cycling cellular states, such as the cell cycle (17, 18). On top of this, we may expect different cellular states to affect the type and level of soluble factors secreted by individual cells (19) and how other individual cells respond to them (20, 21). Because the activity and state of these diverse cellular processes influence each other and are determined by—but also define how—cells spatially organize themselves, there exists extensive feedback at both the single-cell and the cell population level.

Remarkably, although a large amount of knowledge exists for individual cellular processes, very little is known about how they act together to determine cell-to-cell variability. Specifically, quantitative assessment of how

information flows from one cellular property to the next is almost completely absent. I expect that a particularly intriguing class of molecular regulators, namely those that control and establish cell-to-cell variability, will emerge and become important for studying this. When such regulators are perturbed, there may be no change in overall cellular activity, but the pattern of cell-to-cell variability of the activity will be different (22). These regulators could represent an entirely missed concept in molecular cell biology. Another fascinating topic will be the molecular mechanisms by which cells control their intrinsic variability. For clathrin-mediated endocytosis, we have shown that cells that are sparsely growing display more cell-to-cell variability than cells growing in more crowded regions (5). This suggests that local cell density can regulate intrinsic variability.

Finally, hypothesis-driven statistics and causal inference from large sets of accurate measurements may allow us to overcome one of the greatest problems in molecular cell biology: the fact that genetic and pharmacological perturbations have a variety of indirect effects, obscuring the fine detail of the molecular causality underlying cellular activities. Ironically, it is exactly the biologically determined variability in such measurements, whether of single cells in a population or subcellular structures within a cell (23), that will enable us to reveal this.

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GEOCHEMISTRY

A Hard Life for Cyanobacteria

Robert Riding

Many organisms precipitate minerals (1), and sometimes the reasons appear obvious. Humans, for example, rely on biominerals to provide vital skeletal support. But interpreting biomineralization can also be challenging. On page 459 in this issue, Couradeau *et al.* (2) show that the cyanobacterium *Candidatus* Gloeomargarita lithophora from Alchichica, an alkaline lake in Mexico, produces the amorphous mineral benstonite. This rare carbonate has a more complicated composition than common calcium carbonate minerals such as calcite. Furthermore, the particular variety of benstonite produced by the bacterium contains almost as much barium, magnesium, and strontium as calcium. But the point that focuses attention is that the benstonite occurs inside the bacterial cells.

Intracellular inclusions of various types are widespread in bacteria (3). Most do not contain minerals, but there are some well-known examples that do, including magnetic iron crystals that aid orientation in magnetotactic bacteria, and granules that store sulfur in sulfur bacteria (3). However, intracellular calcification has been unknown until now in cyanobacteria. As Couradeau *et al.* show, the benstonite inclusions in *Gloeomargarita* are spherical and less than 0.5 μm across. The cells themselves are only a few micrometers long; some are packed with inclusions. The authors suggest that the cells and their biomineralization may have previously been overlooked because of their small size and possibly also their scarcity.

Extracellular calcification in cyanobacteria has long been recognized (4). It typically involves calcite crystals that can form on the cell surface (5) but most commonly, and most durably, impregnate the protective mucilaginous sheath around the cells (6). The sheath structure itself may play a role in the calcification process (4), but two environmental influences are crucial: the carbonate saturation state of the adjacent water, which affects precipitation of calcium carbonate minerals (7, 8), and the availability of dissolved carbon dioxide, which affects photosynthesis (5, 9). When carbon dioxide levels are low, pres-

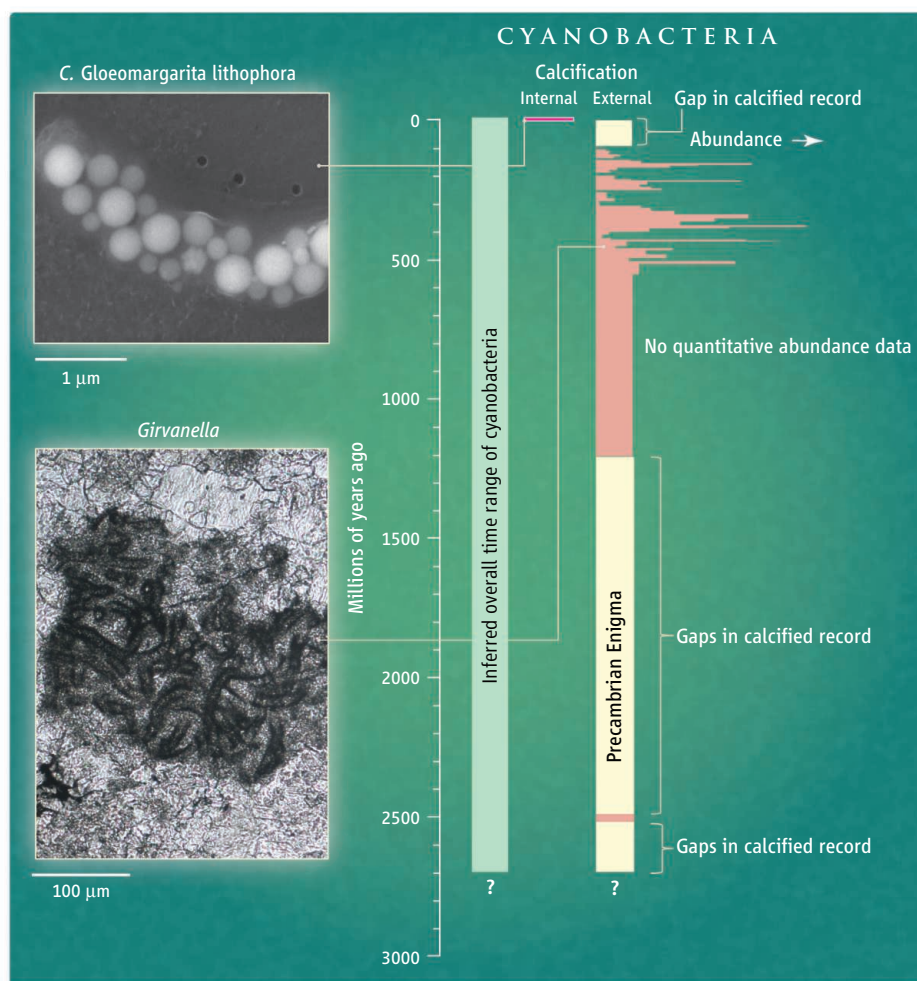
ent-day cyanobacteria turn to other sources of dissolved inorganic carbon, such as bicarbonate, for photosynthesis. This requires conversion of the bicarbonate to carbon dioxide, which raises pH just outside the cells and promotes calcification if water chemistry favors precipitation (6).

To account for calcification in *Gloeomargarita*, Couradeau *et al.* also infer a key role for photosynthesis. But they suggest that the excess alkalinity produced by photosynthesis is trapped in the cell by the benstonite inclusions, rather than being exported as in the case

The discovery of calcification inside present-day cyanobacteria raises questions about their geologic record.

of sheath calcification. The authors argue convincingly that both the intracellular location of the benstonite and its composition, which differs from that of other carbonate minerals deposited in Lake Alchichica, suggest a degree of cellular control over the biomineralization process. They hypothesize that the benstonite granules could act as ballast to help *Gloeomargarita* live at the bottom of the lake.

The discovery of a cyanobacterium with intracellular mineral inclusions may help to understand the geologic record of cyanobacteria (see the figure). Despite often being



Extracellular and intracellular calcification in cyanobacteria. Organic and silicified fossils, together with evidence for oxygen, suggest that cyanobacteria have existed for at least the past ~2700 million years (green column) (12). Yet sheath-calcified cyanobacterial fossils, exemplified by *Girvanella*, have a very episodic distribution in marine environments (right column); before 1200 million years ago, there is only one recorded example 2500 million years ago (13). Calcified sheath abundance data for the past 550 million years are from (10). Couradeau *et al.* now report evidence for intracellular calcification in *Gloeomargarita* from present-day Lake Alchichica, Mexico (2). Geologic evidence for such internal calcification may, however, be difficult to find.

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less than 20 μm across, calcified cyanobacterial sheaths have a good, but highly episodic, microfossil record (10), reflecting the response of sheath calcification to environmental effects. Well-calcified cyanobacterial sheaths first became conspicuous in the marine geologic record about 1200 million years ago (11), remained relatively common until about 100 million years ago, and today are scarce in seawater but locally abundant in fresh water (6). Their scarcity in rocks older than about 1200 million years has attracted particular attention. It seems that sheath calcification rarely occurred in marine cyanobacteria during the first half of their history. A possible explanation for this “Precambrian Enigma” (6) is that the high carbon dioxide levels during this period meant that cyanobacteria did not need to use bicarbonate (6).

It is not yet known whether the controlled intracellular calcification shown by *Gloeomargarita* has a geologic record. If it does, it might be much less erratic than the one for

calcified sheaths. Couradeau *et al.* suggest that ancient relatives of *Gloeomargarita* may indeed have carried out intracellular calcification. They reason that *Gloeomargarita* has ancestral features and that the alkalinity export involved in sheath calcification might require cellular mechanisms that did not evolve until later.

However, it may be difficult to find geologic evidence. Tiny benstonite inclusions like those in *Gloeomargarita* would not be easy to identify with confidence in Precambrian rocks. Recognizing this difficulty, Couradeau *et al.* suggest that carbonate deposits containing barium and strontium, present in the benstonite, might provide a better geologic indicator for this style of biomineralization than the granules themselves.

Couradeau *et al.*'s discovery raises some intriguing possibilities, but assessing their importance for the fossil record will be challenging. In addition to searching for scarce calcified sheaths, we now need to be on the

lookout for subtle traces of intracellular calcification. This is likely to cause some head scratching. At least we know why our skulls are so well calcified. Or do we?

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MOLECULAR BIOLOGY

Reprogramming the Genetic Code

Jason W. Chin

The genetic code provides rules by which a genome is decoded to produce proteins of defined amino acid composition and sequence. These rules, which specify 61 codons (triplets of nucleotides) that code for the 20 common amino acids, and 3 codons that signal the termination of protein synthesis, are near-universally conserved in living organisms. Despite conservation of this code and the translational machinery that enforces it, a growing body of work addresses the challenges in reprogramming the genetic code. Designer amino acids, created by synthetic chemistry, can now be incorporated into specific sites in proteins of interest in vitro, in cells, and most recently in a whole animal (see the figure). These routes to unnatural polymer synthesis and evolution are already facilitating the study of cellular processes including protein interactions, protein conformational changes, posttranslational modification biology, and the kinetics of protein transport and cell signaling with a new level of molecular precision (1). Emerging develop-

ments may allow the synthesis and evolution of new materials and therapeutics.

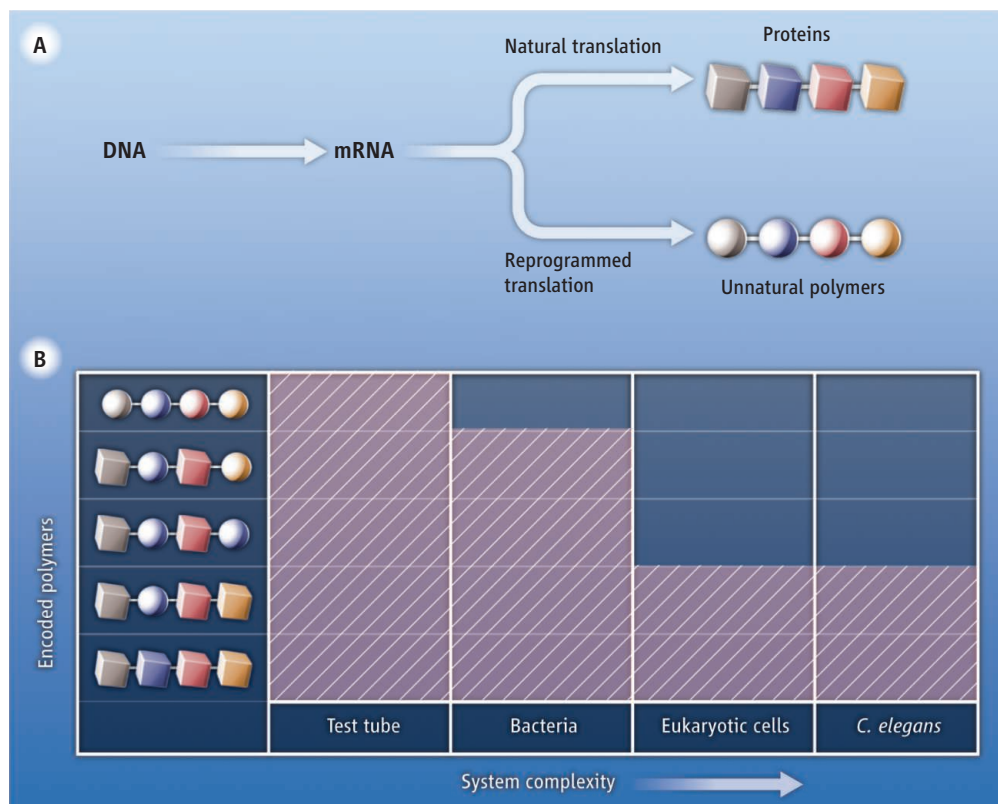
The fidelity of natural protein synthesis is maintained by specific aminoacylation of a transfer RNA (tRNA) with an amino acid, and the ribosomal decoding of each tRNA in response to a cognate codon on a messenger RNA (mRNA) (2, 3). Incorporating unnatural amino acids (with different chemical and physical properties that differ from those of the common amino acids) into proteins requires methods for loading them onto tRNAs and unique codons that can be specifically decoded by “orthogonal” tRNAs, which have been engineered not to be recognized as substrates for endogenous aminoacyl-tRNA synthetases. Chemical methods to aminoacylate orthogonal tRNAs designed to recognize a stop codon (the amber codon), and the use of these tRNAs in translation reactions in vitro, provided the first general route to incorporating unnatural amino acids into proteins (4). Recent advances have expanded the scope of in vitro protein synthesis. For example, quadruplet codons (four nucleotides) that can be read, albeit poorly, on the natural ribosome have been used to incorporate several unnatural amino acids into a single protein

Incorporating unnatural amino acids into proteins presents challenges in expanding the genetic code.

(5). In vitro translation can also be reconstituted from purified factors, allowing particular aminoacyl-tRNA synthetases and tRNAs to be omitted from the translation reaction. The resulting “blank” codons can then be reassigned to new amino acids by introducing orthogonal tRNAs bearing anticodons complementary to the blank codons (6, 7). Ribozymes that catalyze the aminoacylation of tRNAs with unnatural amino acids and other monomers including alpha hydroxy acids (8) have provided an accessible alternative to chemical aminoacylation of tRNAs. This method has allowed the synthesis and directed evolution of unnatural peptides and cyclic peptides bearing a range of unnatural amino acids and monomers (8). Thus far, in vitro approaches have been used to synthesize and evolve antibiotic-like molecules and to label proteins for fluorescence resonance energy transfer experiments (5, 8).

Extending unnatural protein synthesis to cells has also seen progress. The injection of a chemically aminoacylated orthogonal tRNA, engineered to recognize an amber codon, into *Xenopus laevis* oocytes has facilitated new insight into the structure and function of membrane proteins, including

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Unnatural translation. (A) DNA is transcribed to mRNA, and the natural translational machinery decodes the mRNA to produce polymers of the natural 20 amino acids (cubes). Reprogrammed translation allows the introduction of unnatural amino acids (spheres) into proteins. (B) Reprogramming the genetic code in systems of increasing complexity.

Another challenge to increasing the number of distinct amino acids that can be incorporated into proteins in cells is the creation of additional orthogonal aminoacyl-tRNA synthetase-tRNA pairs. The recent de novo generation of such pairs by directed evolution in *E. coli* offers one solution (17).

It has been more than 40 years since in vitro polyester synthesis was demonstrated (through the chemical modification of tyrosine on a tRNA) (18). However, many challenges remain in reprogramming the translational machinery of cells for the encoded synthesis of diverse polymers. Future progress will provide new insight into fun-

damental questions in biology and promises routes to new materials and therapeutics.

ion channels, through the incorporation of unnatural amino acids (9). Orthogonal aminoacyl-tRNA synthetase-tRNA pairs that do not cross-react with endogenous synthetases and tRNAs can specifically recognize a particular unnatural amino acid and efficiently direct its incorporation in response to a unique (usually the amber) codon. Such pairs have now been described in particular host cells (1, 10). The pyrrolysyl-tRNA synthetase-tRNA pairs from *Methanosarcina* species can be used to incorporate unnatural amino acids into protein in bacteria, yeast, mammalian cells, and even a whole animal, the worm *Caenorhabditis elegans* (1). Indeed, the extension of unnatural amino acid-based tools—developed in cells—to plants and animals (11) may ultimately provide approaches for investigating more complex phenomena, such as neural processing and development, with new levels of molecular, spatial, and temporal precision.

Because all the codons are used in the genome for protein synthesis, the incorporation of multiple unnatural amino acids into proteins in cells requires additional blank codons. To address this issue, a parallel and independent or “orthogonal” translation pathway has been created in the model bacterium *Escherichia coli* (12, 13). An orthogonal ribosome, which translates orthogonal mRNAs that are not substrates for the natu-

ral ribosome, has been created. Altered, by directed evolution in the laboratory, it can read quadruplet codons with extended anticodon tRNAs that are poorly decoded on the natural ribosome. This provides a set of blank quadruplet codons that have been assigned to new amino acids by means of orthogonal synthetases and tRNAs, creating a parallel translation pathway in the cell (12, 13). This approach has been used to genetically direct the formation of a nanoscale redox-insensitive cross-link that may be useful in trapping discrete, functional conformations of proteins and creating stabilized protein therapeutics.

Emerging advances in genome engineering (14, 15) may generate complementary approaches to providing additional blank codons. By replacing the codons in the genome with a reduced set of codons that encode all 20 amino acids and deleting the endogenous tRNAs that decode the replaced codons, it may be possible to convert some sense codons into blank codons. The number of blank sense codons that can be independently decoded may be constrained by the extensive wobble pairing rules by which many tRNAs decode several codons. Experimental efforts at codon replacement may reveal the extent to which protein folding and homeostasis, and cellular robustness are evolutionarily embedded in natural codon usage (16).

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IBI* SERIES WINNER

Engaging Undergraduates in Global Health Technology Innovation

Rebecca Richards-Kortum,^{1,2†} Lauren Vestewig Gray,² Maria Oden^{1,2}

"Appropriate Design for Global Health," the IBI Prize-winning module, primes students to respond to global health challenges with novel technological solutions.

It takes only 90 minutes to fly from Miami to Port-au-Prince, Haiti, but the cities are a world apart. A baby born in Port-au-Prince is nearly 10 times as likely to die before age 1 as an infant born in Miami. Life expectancy on the island is almost 20 years shorter than in the United States. That such disparities persist despite renewed public and private commitments to improve global health illustrates the need for a new generation of innovators who recognize the challenges and can design and deploy new health-care technologies that are both highly effective and affordable (1). In response, we and others have developed educational programs to engage globally minded health-care innovators (2). Our approach is inspired by a Haitian saying that captures the essence of inquiry-based education: "You don't learn to swim in the library; you learn to swim in the river."

We created Beyond Traditional Borders (BTB), a program to train undergraduates from all majors to work across disciplinary and geographic borders to design novel technology solutions to real-world global health challenges (3). This curriculum has been institutionalized as a minor in global health technologies at Rice University and has engaged more than 10% of the university's undergraduates in its classes. It has also been adapted for high school students (4).

The introductory inquiry-based module is "Appropriate Design for Global Health." Students learn to use the engineering design process to develop innovative technologies addressing global health challenges. To inform their designs, students learn about important global health issues, the framework to understand factors that limit and facilitate access to health technologies, and the role that new technologies can play in solving global health problems. Students also examine case studies of successful global health interventions (5).

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*IBI, Science Prize for Inquiry-Based Instruction; www.sciencemag.org/site/feature/data/prizes/inquiry/.

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Hit the road. Throughout the design process, students are mentored by health-care providers in the developing world. Exceptional students travel to the developing world to implement their designs in partnership with health-care professionals.

Design challenges are identified by partners who provide health care in low-resource settings. For example, one challenge was, "Develop an accurate method to determine hematocrit that does not require electrical power." Additional challenges can be found in the supplementary materials.

As teams design their solutions, they work to (i) define the problem, (ii) develop a solution, (iii) create and test a working prototype, and (iv) refine and present the solution. Health-care providers and engineers in the developing world and the United States mentor students, and they can revise their work appropriately.

Through literature reviews and communication with mentors, students identify constraints that their solutions must satisfy. Guided by their own problem statements, teams design technologies that can be taken into the field and demon-

Get real. Students design technologies to solve real global health challenges. The efficacy of the student-designed bubble CPAP technology is now being evaluated in clinical trials in Malawi.



About the Authors



Rebecca Richards-Kortum is the Stanley C. Moore Professor of Bioengineering at Rice University. Her research integrates advances in nanotechnology, imaging, and microfabrication to develop point-of-care diagnostics for low-resource settings. Richards-Kortum established Beyond Traditional Borders in 2006. She is a member of the National Academy of Engineering and served on the National Academies Committee on Conceptual Framework for New Science Education Standards.

Maria Oden is a professor in the Practice of Engineering Education and directs the Oshman Engineering Design Kitchen at Rice University. Oden has developed engineering design courses that emphasize open-ended problem solving of real-world design challenges.



Lauren Vestewig Gray is Executive Director of Rice 360°: Institute for Global Health Technologies at Rice University. She helps build international collaborations that have led to internships for more than 60 students and partnerships with high schools around the "Bio-engineering and World Health" curriculum, which has been taught to more than 2000 high school students.



spinner. Laboratory testing showed that the centrifuge is as accurate as a commercially available centrifuge costing 10 times as much (6). Students designed light-emitting diode-based phototherapy lights to treat neonatal jaundice. The lights can be made for less than \$100, whereas the cost of commercially available phototherapy lights can exceed \$6000. An institutional review board (IRB)-approved clinical study in Guatemala found that the low-cost lights are as effective as conventional phototherapy in treating neonatal hyperbilirubinemia (7). Another student designed a portable, battery-operated fluorescence microscope that can be made for \$240. Results of the low-cost microscope for detecting *Mycobacterium tuberculosis* in sputum smears were concordant with those from a laboratory-grade fluorescence microscope in 98.4% of cases (8).

Two student-designed technologies have been distributed broadly in the developing world. The Ministry of Health in Ecuador is using 25 diagnostic labs-in-a-backpack country-wide. About 214,000 "Dose-Right" syringe clips, which fit into the barrel of an oral syringe to ensure accurate dosing of medication, were recently delivered to Swaziland. The clips

are being used by 12,000 participants in the country's national Prevention of Mother-to-Child Transmission of HIV/AIDS program to ensure that infants receive the proper dosage of liquid antiretroviral medication. The technology has been licensed and is now available commercially, with preferred pricing for GAVI Alliance countries (9).

We are preparing a third technology for countrywide dissemination. Two years ago, a team developed a robust, low-cost bubble CPAP (continuous positive airway pressure) system to assist babies in respiratory distress in the developing world. With support from the National Collegiate Inventors and Innovators Alliance and the U.S. Agency for International Development, the system has been refined and is now undergoing an IRB-approved clinical trial in Malawi (see the second photo). One of the students on the

design team is working with physicians in Malawi to implement the trial.

Students participating in the design courses were surveyed to assess whether the course project enhanced their skills. Compared with other civic research or design courses at Rice, a greater fraction of global health technology students reported enhanced skills in creativity, leadership, ability to effect social change, and ability to solve real-world problems. International student interns report that the internship had a strong impact on their career intentions; 95% of interns indicated that they intend to include global health as an important part of their careers.

We have discovered that giving students the opportunity to solve real global health problems not only creates leaders for tomorrow's global health technology workforce but also produces technologies with the potential to revolutionize health-care delivery in poor settings. The shared challenge now for universities and organizations that train students to develop global health technologies is to create a strong pipeline that bridges the divide between student-designed prototype and commercialized product. Only when the path to commercialization is well marked will the tremendous promise of these technologies be realized.

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Supplementary Materials

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strated to health-care providers for feedback during extended summer internships. Technologies are expected to meet all appropriate safety and regulatory standards.

Exceptional students who are selected for internships in the developing world demonstrate and, in some cases, implement the technologies under the guidance of trained health-care providers (see the first photo). Interns are assigned three tasks: demonstrate technologies and gather feedback, develop and implement a solution to another challenge identified by the host site, and pinpoint new design challenges.

Since 2006, BTB students have developed 58 technologies. Many students continue to improve their technologies through independent study. For example, a team designed a hand-powered centrifuge that can be constructed for \$35 using a salad



SOUTHWESTERN AND ROCKY MOUNTAIN DIVISION

On Great Plains, Juniper Invasion Signals Prairies in Distress

TULSA, Oklahoma—When the first Europeans made their way to the Great Plains in the center of the North American continent, they found vast prairies extending to the horizon, with the waves of grass hardly broken by trees. But a visitor to those same prairies today—from Texas up to South Dakota—is likely to find an army of shrubby junipers overtaking the grass.

Far more than an aesthetic change, the shifting ecology places both wildlife and humans at risk: The trees crowd into grazing land and consume water that might otherwise nourish the prairie or provide supplies for local towns and cities. They discharge clouds of highly allergenic pollen and harbor mosquitoes that carry West Nile virus. And they usurp habitat crucial to species such as the lesser prairie chicken and the prairie mole cricket.

“Juniper invasion has emerged as a dominant threat to some of the most threatened ecosystems of North America,” says Samuel Fuhlendorf, a professor in the Natural Resource Ecology and Management program at Oklahoma State University (OSU).

At the recent meeting here of the AAAS Southwestern and Rocky Mountain Division, Fuhlendorf and others detailed how human activity over the past 150 years has upset a natural balance that endured on the prairies for millennia. The question now—for farmers and ranchers, hunters, conservationists, consumers, policy-makers, and

scientists—is whether humans can help restore the balance and the prairie’s health.

The 86th Annual Meeting of the Southwestern and Rocky Mountain Division convened 31 March to 4 April at the University of Tulsa (TU) to explore regional issues with global resonance—cybersecurity, new digital tools for archaeology, research ethics, and the relationship between science and religion, among others. The meeting was held in conjunction with the 15th Annual TU Research Colloquium and the 10th Annual University of Oklahoma-Tulsa Research Day.

Symposia on the juniper invasion and Oklahoma’s endangered species explored the health of the Great Plains, but the lessons apply to grasslands worldwide.

Eastern redcedar (*Juniperus virginiana*) and mountain cedar (*Juniperus ashei*) are native to the region, but for millions of years a natural cycle of wildfires held them in check.

Then, in the 19th century, European pioneers brought intensive cattle-grazing. The sodbusters soon followed, turning up millions of acres of native grass to plant wheat. After the disastrous Dust Bowl of the 1930s, thousands of Eastern redcedar were planted as windbreaks to hold the soil in place. Wildfires were suppressed as a threat to human communities.

Not until it was too late did people realize that the disrupted soil and lack of fires create favorable conditions for the juniper.

March of the junipers. The trees’ aggressive spread “has emerged as a dominant threat to some of the most threatened ecosystems of North America,” says one researcher.

In 2002, the U.S. Department of Agriculture estimated that 8 million acres in Oklahoma alone were “infested” with 50-plus junipers per acre. The conquest was growing by 762 acres a day, doubling every 18 years. New birds follow the trees into the prairie; they eat the juniper berries and expel the seeds, aiding the invasion with each meal. Rising levels of atmospheric carbon dioxide, associated with climate change, may also be fueling the advance, researchers told the AAAS division audience.

Researchers see the lesser prairie chicken as symptomatic of the plains’ stressed condition. Agriculture, overgrazing, and energy development have helped reduce its population by 90% since 1900. Fuhlendorf says that junipers and wind turbines pose similar threats: The bird “tolerates basically no vertical structures,” he said, because it perceives them as haven for predators.

The impact of juniper proliferation is felt even in urban centers. Estelle Levetin, a TU aerobiologist, reported that eastern redcedars around Tulsa appear to be driving “a significant increase” in airborne juniper pollen. Early results in a study covering 25 years suggest a 110% increase in the city’s springtime pollen, she said.

Other research indicates that the junipers disrupt the prairie water cycle. Mature trees may consume up to 35 liters of water a day. Don Turton, a forest hydrologist at OSU, said that preliminary research results attribute a 20% average net loss of water to redcedar canopies. That leaves soil drier and reduces the flow of surface streams. In heavily infested watershed areas along the Canadian River, that may reduce water supplies for Oklahoma City.

In an arid state like Oklahoma, water is a rallying point for diverse interests, from ranchers and farmers to hunters, anglers, and environmentalists. Over the past decade, campaigns to control the junipers have intensified in many parts of the Great Plains. Some are working to conserve blocks of native grassland and reintroducing herds of buffalo. Others have established cooperatives to manage controlled burns, using new science to help bring an ancient balance back to the prairie.

The Payoff of Federal R&D: iPod, Google, and Human Genome Project

With the U.S. budget under intense pressure, lawmakers increasingly are looking to save money by cutting federal spending on research and development. But at two Capitol Hill briefings, experts offered compelling evidence that that federal investment can yield enormous dividends and warned that cuts to R&D could hinder future economic growth.

At the briefings, co-organized by AAAS, they pointed to the Google search engine, iPod technology, and the Human Genome Project as examples of how federal funding has aided transformational innovation and economic growth. Especially when economic stresses are acute, they said, the nation must maintain its longstanding commitment to invest in future prosperity.

"We have very powerful evidence of the productivity" of R&D spending, said University of California-Davis sociologist Fred Block. "When we're cutting back on that R&D spending, we are eating our seed corn, and upsetting the possibilities of future economic growth and development."

Block joined a panel of experts at the 16 March briefings, organized by the University Corporation for Atmospheric Research and AAAS, and cosponsored by eight other scientific societies. In discussions moderated by Vijay Vaitheeswaran, a senior correspondent for *The Economist*, they grappled with some of the challenges facing the United States as stress on R&D budgets and competition from abroad increases.

Under President Barack Obama's 2013 budget proposal, R&D investment would increase by 1.2% over 2012 funding levels.

Nondefense R&D spending would increase by 5.1% over last year's budget, according to the AAAS R&D Budget and Policy Program, while the proposed defense R&D budget would shrink by 1.9%. The House of Representatives' 2013 budget resolution would reduce discretionary spending further, according to analyses by AAAS. The House proposal would cut nondefense R&D spending by 8% compared to the president's budget request, or 5% below the funding levels approved for 2012.

The prospect of tighter federal funding comes as the focus of U.S. innovation is shifting away from large corporations to universities and smaller start-up companies, and technologies are converging in unexpected ways across disparate fields. These trends point to an increasingly important role for federal R&D spending, the panelists said, since federal programs already support significant cross-disciplinary work at public laboratories and universities.

While they agreed that it is difficult to come up with clear-cut quantitative measures of the impact of federal R&D, data compiled by the U.S. Bureau of Labor Statistics offer some perspective. The data show that the output per unit of labor in the U.S. economy from 1948 to 2007 grew at an annual rate of 2.5%, with 58% of the growth attributed to the increase in knowledge that comes from R&D investment.

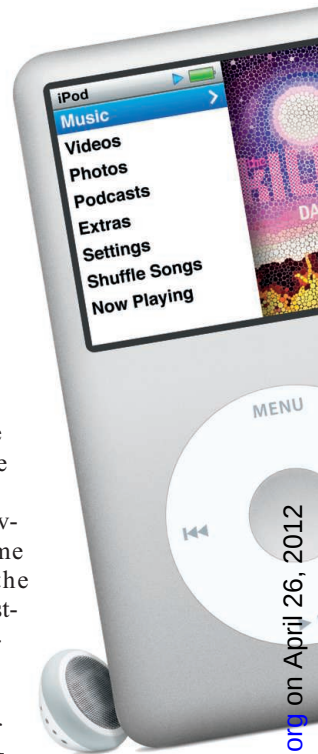
The investment in knowledge represented by the federally funded Human Genome Project had a \$796.3 billion economic impact from 1998 to 2010, according to a 2011 study by Battelle Memorial Institute. Simon Tripp,

senior director of Battelle's Technology Partnership Practice, said that CEOs of major bioscience companies interviewed for the study agreed that without the federal project, "we would be nowhere near where we are today."

This decade's equivalent of the genome project may be the renewed federal investment in energy innovation. Catherine T. "Katie" Hunt, the R&D director for innovation sourcing and sustainable technologies at The Dow Chemical Company, described several Dow projects that have benefited from federal funding, including solar shingles, lithium-ion batteries for electric and hybrid cars, and lightweight carbon fiber blades for next-generation wind turbines.

History suggests that great countries, like great companies, invest during an economic downturn, Vaitheeswaran said. The United States can reap future economic benefits, he suggested, by building on historical strengths in science and technology education and "reinvesting in our base of R&D that fuels the innovation fire."

—Earl Lane and Becky Ham



EDUCATION

AAAS Project 2061 Site Debuts Online Science Tests

Middle and high school teachers can now test their students' knowledge of key science concepts at an innovative Web site developed by Project 2061, AAAS's science literacy initiative.

The "Create & Take Tests" module was added in March to the AAAS Science Assessment site (<http://assessment.aaas.org>), which contains more than 700 test questions on topics from evolution and natural selection to atoms, molecules, and states of matter. Teachers can use the new module to build multiple-choice tests

from items that they select from the site's full database of questions.

The tests can be administered and scored online, providing quick feedback for instructors, said George DeBoer, the deputy director of Project 2061 who led the assessment project. "Getting reliable and timely information about what students know or don't know means that teachers can adjust their instruction to respond quickly to their students' needs."

Josh Flory, an 8th-grade science teacher

at New Albany Middle School in Ohio, has used the feature to design pre- and post-tests for his students in biology, geology, and the physical sciences. The immediate feedback from the online test, he said, "has been a very powerful tool for students, and gives them a chance to see where they're starting from and where they can grow."

Project 2061 developed the assessment items and collected data on them in a 7-year effort funded by a grant from the U.S. National Science Foundation. The site has logged 12,000 registered users and nearly 70,000 visitors since its launch in April 2011.

—Becky Ham

Multiblock Polymers: Panacea or Pandora's Box?

Frank S. Bates,^{1*} Marc A. Hillmyer,² Timothy P. Lodge,^{1,2} Christopher M. Bates,³ Kris T. Delaney,⁴ Glenn H. Fredrickson^{4,5}

Advances in synthetic polymer chemistry have unleashed seemingly unlimited strategies for producing block polymers with arbitrary numbers (n) and types (k) of unique sequences of repeating units. Increasing (k, n) leads to a geometric expansion of possible molecular architectures, beyond conventional ABA-type triblock copolymers ($k = 2$, $n = 3$), offering alluring opportunities to generate exquisitely tailored materials with unparalleled control over nanoscale-domain geometry, packing symmetry, and chemical composition. Transforming this potential into targeted structures endowed with useful properties hinges on imaginative molecular designs guided by predictive theory and computer simulation. Here, we review recent developments in the field of block polymers.

Block polymers, hybrid macromolecules constructed by linking together discrete linear chains comprising dozens to hundreds of chemically identical repeating units, spontaneously assemble into exquisitely ordered soft materials (*1*). Precise synthesis of these self-assembling compounds offers extraordinary control over the resulting morphology, spanning length scales from less than a few nanometers to several micrometers, enabling a diverse and expanding range of practical applications in, for example, drug delivery (*2*), microelectronic materials (*3*), and advanced plastics (*4*). Yet, only a small subset of the vast array of feasible molecular architectures has been explored, and just a handful of these compounds have been developed into commercial products. Combining more chemically distinct blocks and block types, beyond the established AB and ABA diblock and triblock copolymers, offers unparalleled opportunities for designing new nanostructured materials with enhanced functionality and properties, often without adding substantially to the cost of production. But how do we choose among the myriad molecular design possibilities?

Modern synthetic methods afford access to a broad portfolio of multiblock molecular architectures, as illustrated schematically in Fig. 1. (Although there is no universal definition of the minimum molar mass that constitutes a polymer block, generally 10 to 20 monomer repeat units, and frequently more than 100, are used). Linear AB diblock copolymers have been investigated most extensively, leading to a comprehensive ex-

perimental and theoretical understanding of their bulk- and solution-phase behavior (*1*). Extension to linear alternating multiblock copolymers (ABA, ABABA, etc.) can lead to profound consequences on the physical properties (for example, enhanced elasticity and fracture toughness) (*5*) without drastically influencing the associated phase behavior. Introduction of a third block type, C, dramatically expands the spectrum of accessible nanostructured morphologies or "microphases." Whereas AB and ABA copolymers typically adopt four familiar microphase structures (lamellae, double gyroid, cylinders, and spheres), many more ordered phases have been documented with ABC triblock terpolymers (*1*, 6–9). Adding additional numbers of blocks (n) and chemically distinct block types (k) rapidly expands the number of unique sequences (see Box 1), each capable of producing a host of nanostructures. Cyclic and branched architectures further ex-

pand the possibilities (*10*), leading to dizzying phase complexity.

Block polymer phase behavior is determined by a suite of molecular variables, in addition to the molecular topology and specific block sequences, as summarized in Table 1. Primary factors include the degree of polymerization of each block, N_i , and the associated binary segment-segment interaction parameters, χ_{ij} (where i and j refer to chemically distinct repeat units) (see Box 2). (Alternatively, the composition $f_i = N_i/N$ and overall molecular size $N = \sum N_i$ can be used in place of defining each block length, where a common segment volume defines N_i). Secondary factors (not discussed further) include block flexibility (for instance, stiff versus flexible chains), the distribution in block lengths (dispersity), and any additional sub-block structure such as alternating, random, or tapered sequences of repeat units. Obviously, increasing n and/or k even slightly, compounded by scores of practical options in choosing the block chemistries, results in an expansive parameter space and a

Table 1. Molecular variables that influence block polymer self-assembly.

Primary
Topology; linear, branched, etc.
Number of blocks, n
Number of block types, k
Block degree of polymerization, N_i
Interaction parameters, χ_{ij}
Secondary
Block flexibility, b_i
Molar mass distribution, $\bar{D}_i$
Sub-block structure (e.g., tapered)
Composition heterogeneity

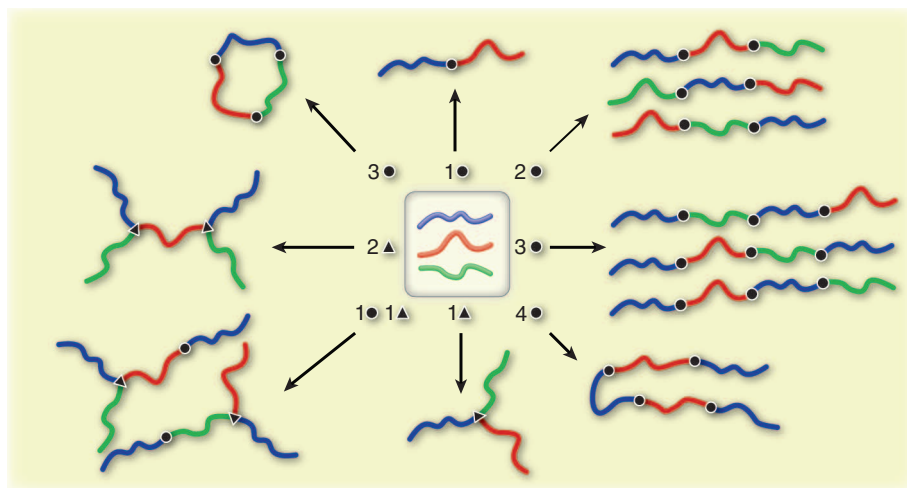


Fig. 1. A subset of the vast structural complexity with two ($k = 2$) or three ($k = 3$) block types produced by varying the number of blocks (n) and the functionality of the connector at each block-block juncture (difunctional, circles; trifunctional, triangles). The number and type of connectors needed are given for each structure.

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Box 1. Ménages en blocs.

The challenge of enumerating possible block polymer structures is already evident in Fig. 1, where it is seen that a substantial number of molecules can be constructed by linking multiple block species using difunctional or trifunctional linkers. An appreciation for the numbers involved can be obtained by considering just the set of linear molecules composed of n blocks, selected from k different species, and connected into a chain by means of $n - 1$ difunctional linkers. The enumeration of all such linear molecules, subject to the restrictions (i) that adjacent blocks along each chain are of different species, (ii) only topologically distinct sequences are counted (i.e., AB is not distinguishable from BA), and (iii) all k block species appear, is a problem in combinatorial analysis—*ménages en blocs*—that is taken up in the supplementary material.

Ménages en blocs has close connections to classic problems in permutations with restrictions, including the 19th century “problème des ménages” (47, 48), which asks for the number of ways of seating n married couples at a circular table, men and women in alternate positions, no wife next to her husband. The blocs problem is also related to problems involving enumeration of protein sequences (49, 50), although in the latter there are normally no species restrictions on adjacent amino acid residues. The restriction (i) for multiblock polymers amounts to the assumption that blocks do not acquire size dispersity by virtue of sequence. Though this assumption is violated for some classes of block polymers formed by statistical linking of monomers and/or preformed blocks by various (e.g., condensation) chemistries, such nonideal systems are beyond the scope of the present discussion.

The object of interest in *ménages en blocs* is $Z(k, n)$, the number of linear block polymers that can be formed from n blocks and k different block species, and is subject to the restrictions (i) to (iii) stated above. This object can be constructed by exclusion from a simpler function $Q(k, n)$ that is defined as the number of linear block polymers that can be formed from n blocks selected from k species and subject only to the restrictions (i) and (ii). An expression for Q is derived in the supplementary material

$$Q(k, n) = \begin{cases} \frac{k}{2}(k-1)^{(n-1)} & n \text{ even} \\ \frac{k}{2}(k-1)^{(n-1)/2}[1 + (k-1)^{(n-1)/2}] & n \text{ odd} \end{cases}$$

Imposing the final restriction (iii) that all k block species must appear in each molecule is trivial for the case of $k = 2$, because restriction (i) implies restriction (iii); hence, $Z(2, n) = Q(2, n)$. For $k > 2$, the principle of exclusion is used to recursively generate $Z(k, n)$ from $Q(k, n)$

$$Z(k, n) = Q(k, n) - \sum_{j=2}^{k-1} \binom{k}{j} Z(j, n)$$

In words, block polymers containing $k - 1$ or fewer block species are removed from the set comprising $Q(k, n)$ to yield $Z(k, n)$, the subset of molecules in $Q(k, n)$ in which all k block species appear.

Application of the above formulas leads to notable growth in block polymer diversity with increasing k and n . The table below summarizes the $Z(k, n)$ function for block enumerations with $k, n \leq 10$. One observes that there are no molecules with $k > n$, as is required by restriction (iii). For $k = 2$, there is an odd-even effect with increasing n (e.g., the ménage à trois ABA and BAB at $n = 3$, ABAB at $n = 4$). Along the diagonal, block polymer complexity explodes in a factorial manner with $Z(n, n) = n!/2$.

Ménages en blocs function $Z(k, n)$

k/n	2	3	4	5	6	7	8	9	10
2	1	2	1	2	1	2	1	2	1
3	0	3	9	24	45	102	189	402	765
4	0	0	12	72	300	1092	3612	11,664	36,300
5	0	0	0	60	600	3900	21,000	102,120	466,200
6	0	0	0	0	360	5400	50,400	378,000	2,502,360
7	0	0	0	0	0	2520	52,920	670,320	6,667,920
8	0	0	0	0	0	0	20,160	564,480	9,313,920
9	0	0	0	0	0	0	0	181,440	6,531,840
10	0	0	0	0	0	0	0	0	1,814,400

Curiously, the maximum of $Z(k, n)$ for $n > 4$ is above the diagonal, located at $Z(n - 1, n)$ for $5 \leq n \leq 8$, and at $Z(n - 2, n)$ for $n = 9, 10$. Synthesis of all these distinct linear block polymer molecules will surely keep synthetic polymer chemists busy for some time!

boundless array of possible structures and chemical functionalities.

Multiblock polymers, such as those sketched in Fig. 1, offer unlimited potential for designing soft materials with precisely specified structures, subject to two critical limitations: (i) The structures can be accessed synthetically, and (ii) predictive theoretical tools to guide molecular design are available. A parallel can be drawn with the complexity and diverse functions associated with proteins, another well-known class of multifunctional macromolecules. Theorists strive to predict polypeptide structure and ultimate function based on models that employ quantum chemical and statistical mechanical tools capable of accounting for both short-range (~ 0.1 nm) and long-range interactions mediated by an aqueous environment and constrained by the macromolecular architecture (11). Essentially any sequence of less than 100 residues of the 20 naturally occurring amino acids (and a bevy of unnatural variants) can be produced using commercially available solid-phase peptide synthesizers. The challenge is to anticipate which linear combinations of amino acids, drawn from 20^n possibilities, will lead to the desired secondary, tertiary, and even quaternary structures and corresponding functions.

In some respects, block polymers appear to pose a less daunting theoretical challenge, particularly for flexible and noncrystalline polymers, because the chain structure at the monomer level does not play a primary role in determining the domain geometry or packing symmetry. Moreover, with no direct analog to solid-phase peptide synthesis, contemporary research focuses on producing block polymers with a minimum degree of complexity (that is, the smallest n and k) necessary to achieve a desired morphology and complement of properties. However, whereas protein folding is intrinsically intramolecular, supramolecular self-assembly of block polymers leads to ordered structures with unit cells that may contain many thousands of polymer chains (millions of atoms), resulting in unique theoretical and computational challenges. Hence, even the seemingly primitive case of ABC triblocks ($k = 3$, $n = 3$) is only marginally understood in terms of the resulting phase structure and associated properties.

Synthesis

Block polymer synthesis has evolved considerably since the introduction and development of living anionic polymerization by Szwarc more than 50 years ago (12). Numerous strategies that enable the covalent connection of two or more polymer blocks have been developed, including sequential addition of distinct monomers to an active polymer chain, chain-coupling strategies, and transformations of polymer end groups to accommodate diverse and often incompatible polymerization mechanisms. Chemical connectors that link together more than two chains are increasingly available as evidenced by the heroic syntheses of a 31-arm starblock pentapolymer

Box 2. Complexity with χ .

Discussions of polymer phase behavior—whether block polymers, blends, or solutions—are usually couched in terms of the binary interaction parameter χ_{AB} , or simply χ . Conceptually, χ is a dimensionless measure of the energetic cost of exchanging a repeat unit of polymer A for an equal volume (V_{ref}) unit of polymer B: $\chi = z\Delta w/kT$, where kT is the thermal energy (k is the Boltzmann constant, and T is temperature), Δw is the difference between the A/B interaction energy w_{AB} and the average of the self-interaction energies w_{AA} and w_{BB} , and z is the number of nearest neighbors. On the assumption that there is entirely random mixing of A and B units, with entropy arising only from the ideal combinatorial of mixing $\Delta S_m^{\text{id}} = -k\sum(\phi_i/N_i)\ln\phi_i$ (where ϕ_i and N_i are the volume fraction and degree of polymerization of species i , respectively), the Flory-Huggins (or Bragg-Williams or mean-field or regular-solution) free energy of mixing for A and B polymers becomes

$$\Delta G_m = -T\Delta S_m^{\text{id}} + \chi kT\phi_A\phi_B$$

However, in experimental practice, χ is imbued with whatever attributes are needed to describe the data; as such, it actually represents an excess free energy of mixing ΔG_m^{ex} that exhibits both enthalpic and entropic contributions. Therefore, in practice χ is recast as $\chi = \chi_H + \chi_S = \alpha/T + \beta$ (where α and β are empirical parameters), and either α or β may also depend on ϕ and N . This complexity can lead to much confusion when, for example, comparing different measurements on the same system. Furthermore, either α or β may be positive or negative in sign. The case where $\alpha > 0$ and $\beta < 0$ leads to the “classical” upper critical solution temperature phase diagram, whereas $\alpha < 0$ and $\beta > 0$ leads to a lower critical solution temperature (i.e., phase separation on heating); both situations are common. Because ΔS_m^{id} varies inversely with N , it is negligible for typical molecular weights; consequently, the excess free energy is not just a correction, but the main contribution.

The characteristic magnitude of χ_{AB} spans several orders of magnitude, as illustrated below. From a predictive point of view, the situation is quite unsatisfactory. For systems in which short range, isotropic, dispersive interactions dominate, the solubility parameter approach might be expected to yield reasonable estimates [i.e., $\chi \approx \chi_H = V_{\text{ref}}/kT(\delta_A - \delta_B)^2$, where δ_A and δ_B are the associated segment solubility parameters]; however, in practice, agreement with experiment is often remarkably poor. Additionally, although solubility parameters can be used to anticipate qualitatively where a particular blend might lie on this continuum, there is no general theoretical approach for predicting the relative contributions of χ_H and χ_S or for computing either term to within, say, a factor of 2. As phase boundaries in polymer systems typically depend on the dimensionless product χN , it is therefore not possible to even anticipate the molecular weights needed to produce an experimentally accessible phase boundary.

Blends of hydrogenous and deuterated isotopomers represent the simplest case. Here, it is usually found that $\chi \leq 10^{-3}$, and χ_H can be understood in terms of differences in zero point energy and the polarizabilities of C–H and C–D bonds. Nevertheless, χ_S is not negligible, for reasons that remain elusive. For example, for (H/D) poly(styrene) blends, χ (373 K) ≈ 0.00014 , but χ_S is of comparable magnitude to χ_H and is negative (51). For poly(styrene)/poly(methylmethacrylate), a pair of prevalent thermoplastics, χ (373 K) ≈ 0.038 , a relatively modest value; however, χ_S is positive and, surprisingly, represents $\sim 70\%$ of the total, so that χ itself has almost no T dependence (52). When polar or ionic polymers are mixed with nonpolar partners, χ can exceed unity. Here, the fundamental interactions are relatively strong, long-ranged, anisotropic, and/or non-pairwise additive, yet entropic contributions still play a substantial role. For example, for poly(styrene)/poly(lactide), χ (373 K) ≈ 0.15 , but the magnitude of χ_S at this temperature is about half that of χ_H (53).

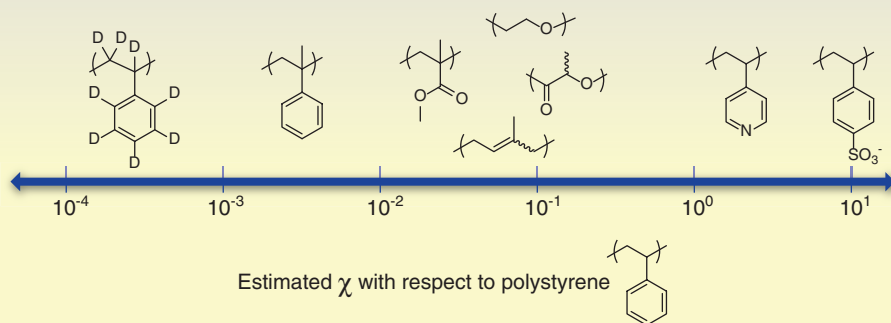
($k = 5$, $n = 31$) (13). In principle, all of the structures depicted in Fig. 1 could be prepared with arbitrary choice of block constituents using modern polymerization methods and polymer functionalization strategies, including numerous controlled radical, ring-opening, metal-catalyzed, and ionic polymerizations, often in combination with highly selective and efficient functional-group transformation techniques (14, 15).

Modern block polymer molecular design is driven by the intended applications. For example, a thermally stable and mechanically robust membrane endowed with a dense array of specifically sized nanopores necessitates a combination of glassy, ductile, and chemically etchable (or cleavable) blocks (16). Biocompatibility further limits the possible ingredients. The associated segment-segment interaction parameters and optimal block architecture demand a carefully targeted synthetic strategy, possibly including multiple routes to the desired structure, drawn from an ever-expanding synthetic tool kit. Although high degrees of molecular uniformity are generally desirable, in most circumstances some level of imperfection (e.g., less than n blocks) and modest chain-length distribution can be tolerated [in fact, tailored dispersity represents a strategic design parameter (17, 18)]; the goal is to build the appropriate structure in the most synthetically economical way possible.

Figure 2 illustrates routes to all possible sequences of a linear ABC terpolymer ($k = 3$, $n = 3$) designed to incorporate three complementary physical and chemical properties: (i) glassy poly(styrene) (PS), (ii) rubbery poly(isoprene) (PI), and (iii) chemically etchable and biorenewable poly(lactide) (PLA). Although only two of these three structures have been reported, PI-PLA-PS is certainly tractable on the basis of a related precedent (19).

The synthetic strategies depicted in Fig. 2 require various combinations of ring-opening, anionic, and controlled radical polymerizations with concomitant end-group manipulations. In fact, the drive to larger n and k will almost certainly necessitate multiple polymerization mechanisms and functional-group manipulations, the order of which will depend on the desired block sequence. Complexity in ABC terpolymer systems is further increased by introducing the possibility of cyclic motifs and three-point junctions (miktoarm stars or brushes). Putting all the pieces together requires the continued development of efficient processes and realizable retrosynthetic analyses.

Introduction of a fourth block with only three monomers ($k = 3$, $n = 4$) provides an added level of complexity not realized in ABC terpolymers. Asymmetric ABCA' tetrablock terpolymers ($N_A \neq N_{A'}$) offer unique opportunities for designing bulk materials (highlighted in the Structure section) and tailored solution structures (20). However, independent control over the length of all four blocks requires synthetic strategies that depend on the specific block chemistries. For



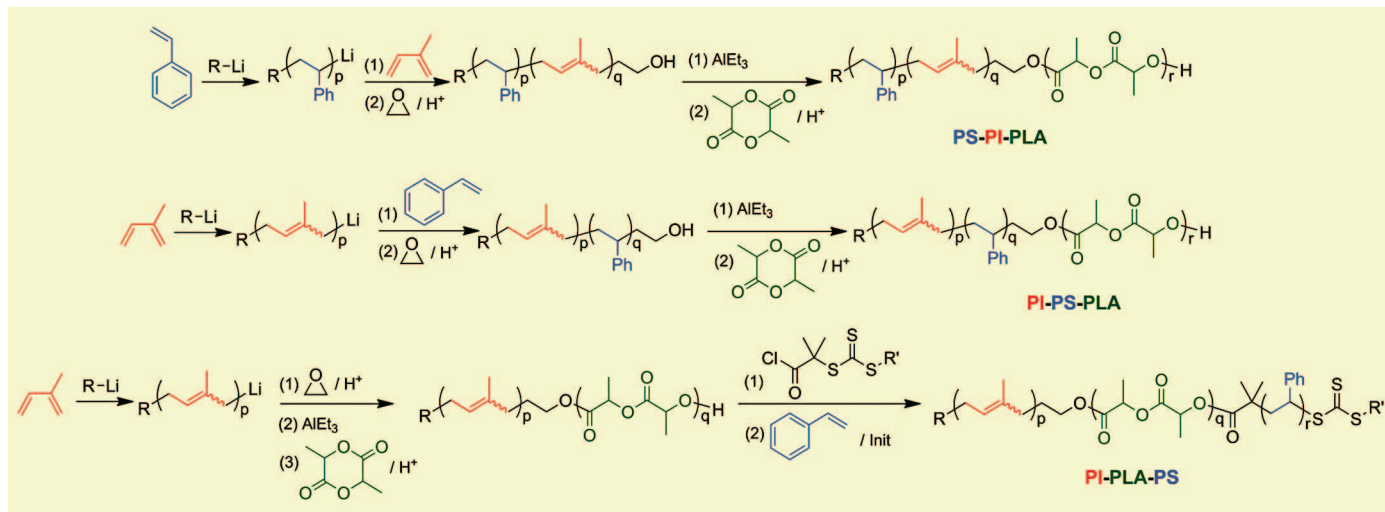


Fig. 2. Synthetic routes to produce the three distinct linear triblock terpolymer structures composed of PS, PI, and PLA blocks, using combinations of living anionic, metal-catalyzed ring-opening, and revers-

ible addition-fragmentation chain transfer-controlled radical polymerizations. R and R', alkyl groups; Ph, phenyl; p, q, and r, degrees of polymerization; Et, ethyl.

example, the addition of water-soluble and biocompatible poly(ethylene oxide) (PEO) to both ends of a PS-PI core should be feasible. Sequential anionic polymerization of styrene and isoprene initiated with an alkyllithium reagent containing a protected hydroxyl group (21) followed by end-capping with one unit of ethylene oxide generates a heterotelechelic diblock copolymer. Activation of the free alcohol end enables the ring-opening polymerization of ethylene oxide, without compromising the protecting group at the other chain end, leading to a linear BCA' triblock; the terminal end of the A' block must be capped to prohibit further growth during subsequent polymerization of ethylene oxide from the B segment in this scheme. Unmasking and activation of the hydroxyl group at the B terminus allows for independent control of N_A relative to $N_{A'}$. Such asymmetric ABCA' tetrablock polymers have not been reported, but this approach could enable remarkable tunability of ordered microstructures (see below).

New organocatalytic polymerization methods (22), controlled synthesis of conducting polymers (23), and various “click” strategies (14), all developed over the past decade, exemplify how advances in chemistry have allowed access to hybrid structures that simply could not be made before. However, deciding what macromolecular “masterpiece” to synthesize using the expanding palette of monomer “paints” and polymer synthesis “brushes” represents a growing conundrum. Targeting new block structures for purely aesthetic reasons is impractical. Today, synthetic polymer chemists can prepare nearly any architecture with any set of desired chemistries, for commodity and value-added applications, paralleling the synthesis of small molecules for therapeutic markets. Organic chemists can build complicated small-molecule structures with an amazing, almost arbitrary array of chemical functionalities;

to know which molecule is efficacious requires a better and more complete understanding of biological action. Analogously, given the heavy investment required for creating even a single new multiblock structure (24), advances in the block polymer/soft materials arena require (i) a deeper understanding of how block architecture influences structure and, thus, properties and (ii) advances in predictive theories that can guide synthetic chemists.

Structure

The fundamental principles governing block copolymer self-assembly were established in the 1970s, culminating in Helfand's strong segregation (25) and Leibler's weak segregation (26) analyses of AB diblock copolymers. These theories have since been subsumed into a comprehensive mean-field framework known as self-consistent field theory (SCFT) (see the Theory section). Equilibrium-phase behavior represents a compromise between minimizing unfavorable segment-segment contacts, mediated by χ_{AB} , and maximizing configurational entropy, which is inversely proportional to N and quadratically dependent on the extent of chain stretching relative to the unperturbed state. Simplifying assumptions, including Gaussian chain (random walk) statistics and constant density, enables tractable computational schemes that have accounted for all of the experimentally observed diblock morphologies (27). Increasing n and k introduces additional complexity, greatly compounded by the choice of block sequences along with the other molecular parameters listed in Table 1.

In the limit of strong segregation, multiblock polymers segregate into relatively pure domains separated from neighboring domains by as many as $k(k-1)/2$ distinct and narrow interfaces characterized by interfacial tension $\gamma_{ij} \sim (\chi_{ij})^{1/2}$; strong segregation implies $N_i \approx N_j \gg 10/\chi_{ij}$. Reducing

the individual values of χ_{ij} leads to broader interfaces and, ultimately, mixing of i and j blocks. Thus, an ABC triblock terpolymer ($k=3$, $n=3$) may contain one, two, or three types of interfaces (or none if entirely disordered). For example, the condition $\chi_{AB} = \chi_{BC} \ll \chi_{AC}$ favors two interfaces (A/B and B/C), whereas $\chi_{AB} = \chi_{BC} \gg \chi_{AC}$ often produces the maximum three. The relative magnitudes of χ_{AB} , χ_{BC} , and χ_{AC} , along with N_i , are the primary determinants of the interfacial topology and overall surface areas. Simple extension beyond diblocks to an ABC architecture has yielded more than 30 distinct microphases (7), many of which have been captured by SCFT (28). Branched (e.g., $k=3$, $n=3$ miktoarm) molecular architectures can enforce interfaces between otherwise incompatible blocks, leading to additional structural diversity (29).

Adding just one additional block ($n=4$) while keeping the number of block types fixed ($k=3$) provides a powerful tool for decoupling domain geometry and ordering symmetry, as illustrated in Fig. 3 for the sequence ABCA', one of nine possible tetrablock terpolymer enumerations, where A and A' represent chemically identical blocks containing N_A and $N_{A'}$ repeat units. Here, we focus on a restricted subset of available molecular parameters defined by $\chi_{AB} = \chi_{AC}$ and $(N_A + N_{A'})/2 = N_B = N_C = N/4$ (i.e., constant composition) with two adjustable variables: (i) the magnitude of the interaction parameter χ_{BC} and (ii) the molecular symmetry factor $\xi = N_{A'}/N_A$. First, we consider the symmetric case $\xi = 1$.

With $\chi_{BC} = 0$ and $\chi_{AB} = \chi_{AC} \gg 10/N$, the $\xi = 1$ tetrablock terpolymer will exhibit a two-domain lamellar morphology with equivalent interfaces separating A and A' domains from mixed B/C domains (Fig. 3A). Increasing the segregation strength to $\chi_{AB} = \chi_{AC} = \chi_{BC}/2$ will result in a three-domain lamellar configuration

with energetically equivalent A/B and C/A' interfaces that exactly balance the internal B/C interface (30). Further elevating χ_{BC} will induce changes in the domain geometry that minimize the overall interfacial free energy ($\sum_{ij} A_{ij}\gamma_{ij}$,

where A_{ij} is the i - j interfacial area) subject to entropic penalties associated with packing the blocks into the resulting structures. The sequence of domains sketched in Fig. 3A, radial and axial segregated cylinders followed by a Janus sphere, illustrates phase transitions that capture this effect by reducing $A_{BC}/(A_{AC} + A_{AB})$, the ratio of interfacial surface areas. (We note that this hypothetical sequence of morphologies may be superseded by other morphologies and never realized in practice). Clearly, adding an A' block to the ABC sequence offers specific control over the shape and subdivision of the self-assembled domains.

Interdomain packing can be considerably influenced by the symmetry parameter ξ , as shown in Fig. 3B. We have selected the axially segregated cylindrical domain structure to illustrate this point. Tetrablock terpolymers ($k = 3$, $n = 4$) can produce ordered cylinders containing B and C subdomains, even with $N_A + N_{A'} = N/2$ (31); cylindrical structures at such low matrix compositions are inaccessible with diblock copolymers. For $\xi = 1$, there will be no interdomain axial order because, under the stated conditions, the intradomain structure does not influence the corona chains that control cylinder packing; two-dimensional (2D) hexagonal packing would

be anticipated. However, for $\xi > 1$, the C domains will be decorated with A' blocks, which are longer than the A blocks emanating from the B portions of the cylinder. This arrangement introduces an effective anisotropic interdomain potential that should induce 3D order. The tendency to pair short and long corona chains represents a purely entropic effect, one that minimizes unfavorable chain stretching and compression at constant density. A different cylinder-packing symmetry (for instance, tetragonal) would be required to permit uniform chain packing; neither three- nor sixfold symmetry supports complete in-plane alternation of C and B domains. Such symmetry-breaking also should apply to the Janus sphere geometry (and radial segregated cylinders) creating dipole-dipole interactions, thus offering the fascinating possibility of tailoring domain orientation within specific ordered lattices analogous to spin alignment in magnetic materials (32) [e.g., noncentrosymmetric ferromagnetic ordering (33) of spheres on a body-centered cubic (bcc) crystal as shown in Fig. 3B] and pairing of colloidal Janus particles (34).

Simply changing the sequence from ABCA' to ABA'C while holding all other parameters constant leads to qualitatively different phase behavior. Fixing $\chi_{AB} = \chi_{AC} \ll \chi_{BC}$ leads to spherical (perhaps cylindrical) domains made up of C cores surrounded by shells of A and A' embedded in a matrix of B; hence, $A_{BC} = 0$. In this case, interdomain packing can be influenced by moderating χ_{AB} and ξ . Recent investigations

with poly(styrene-*b*-isoprene-*b*-styrene-*b*-ethylene oxide) (SISO) tetrablock terpolymers ($\chi_{SI} \approx \chi_{SO} \approx \chi_{IO}/4$) have revealed sphere-packing geometries beyond conventional bcc order, including a σ phase (35) and simple hexagonal symmetry (36).

Clearly, tetrablock terpolymers represent the tip of the iceberg in considering opportunities for designing multiblock polymers with tailored domain geometry, connectivity, and packing symmetry. Characterizing the resulting structures requires a suite of complementary techniques—most importantly, real space imaging by transmission electron microscopy (TEM) and reciprocal space assessment using small-angle x-ray scattering and small-angle neutron scattering (SAXS and SANS) (35).

Theory

Marked advances have been made over the past two decades in the development of theoretical and computational tools for exploring complex block polymer assembly. Based on a methodology developed by Edwards (37), a powerful field-theoretic framework has emerged in which coarse-grained, particle-based models of polymer solutions or melts are converted to statistical field theories by the introduction of auxiliary potential fields (38). By this approach, field theory models can be constructed for virtually any type of interacting polymer system, including the multispecies block polymers that are the present focus, and in any statistical mechanical ensemble of interest. The resulting field theories are

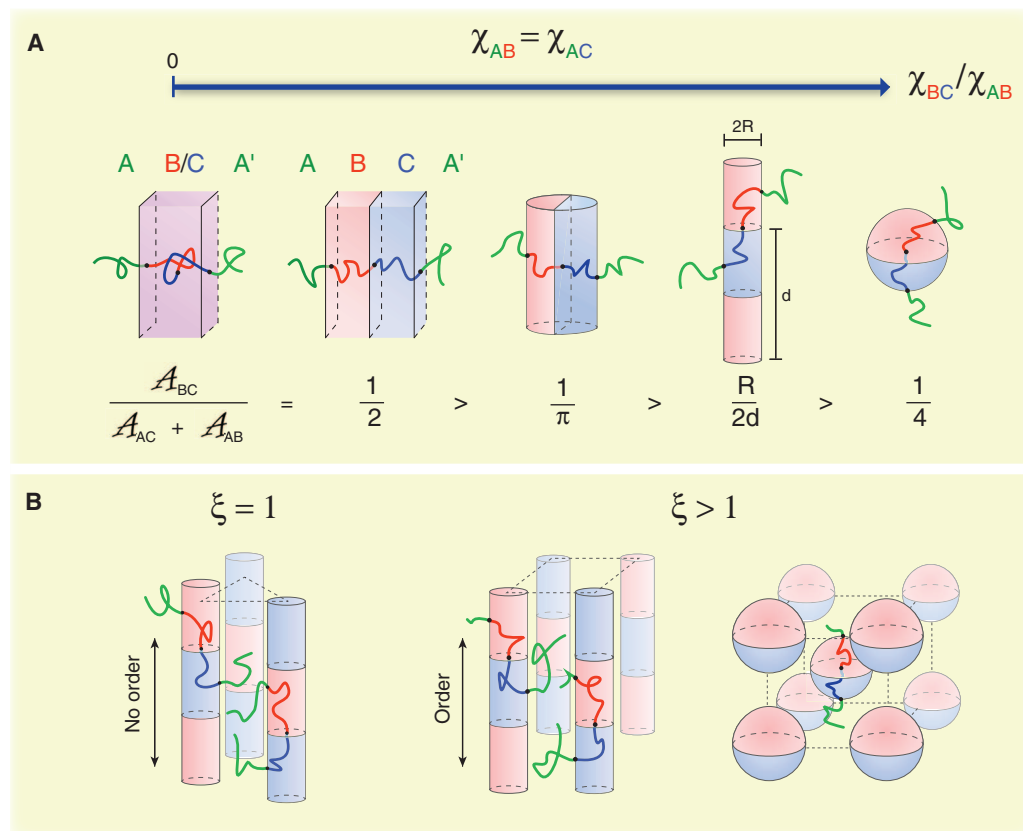


Fig. 3. Hypothetical ABCA' tetrablock terpolymer self-assembly behavior at constant composition, $(N_A + N_{A'})/2 = N_B = N_C$, and $\chi_{AB} = \chi_{AC}$, where $\xi = N_A/N_{A'}$. **(A)** As the ratio χ_{BC}/χ_{AB} increases, the minimization of interfacial energy drives a reduction of the BC interfacial area, A_{BC} , relative to $A_{AC} + A_{AB}$, which produces morphological transitions, from lamella through cylinders to spheres (see text). **(B)** Anticipated domain geometries and packing of ABCA' tetrablock terpolymers as a function of molecular asymmetry. When $\xi = 1$, common A and A' block lengths leads to 2D order (i.e., arbitrary axial registration of the cylindrical subdomains). With $\xi > 1$, short A chains (emanating from the red B domains) will show a preference to pack near long A' chains (emanating from the blue C domains) to maintain constant density while minimizing unfavorable chain stretching and compression. This induces 3D cylindrical order and a ferromagnetic-like arrangement of Janus-type spheres.

characterized by effective Hamiltonians (or actions) H that are highly nonlinear and nonlocal functionals of the field variables and have explicit dependence on segmental interaction and chain architecture parameters, such as χ_{ij} and N_i . The Hamiltonian is generally also a complex-valued functional, which can have important consequences for generating numerical solutions.

Two major classes of field-based simulations can be built on top of the Edwards framework: (i) SCFT (39), where mean-field solutions are sought corresponding to saddle points of H , and (ii) field-theoretic simulations (FTS), referring to stochastic numerical sampling of the full (complex-valued) field theory (40). SCFT simulations are considerably less expensive than FTS, because they seek only a single saddle-point field configuration, but SCFT neglects field fluctuations that are especially important in solvated polymer systems. In the case of high-molar mass undiluted block polymers, such fluctuation effects are substantial only near order-disorder phase boundaries and associated critical points, so SCFT can be applied to polymer melts with relative impunity.

Even within the confines of SCFT, however, establishing the phase map for a given block polymer [for example, from the (k,n) linear multiblock family] can be a daunting challenge. First, the parameter space is large: Even in the case of an ABC triblock, the phase map is defined by a complicated surface within the 5D parameter space $\chi_{AB}N$, $\chi_{AC}N$, $\chi_{BC}N$, f_A ($= N_A/N$), and f_B that has yet to be delineated in detail and would

entail a Herculean effort to complete. Second, the mean-field free-energy landscape explored in SCFT simulations is rough, so simulations launched in large cells from random initial field configurations and subject only to periodic boundary conditions tend to yield defective morphologies that are metastable, rather than stable (lowest in free energy) for the specified parameters. An example is shown in Fig. 4, where such a large-cell SCFT simulation is conducted for an ABC triblock melt using parameters (28) that coincide with a PI-PS-PEO system known to have a stable orthorhombic $Fddd$ (O^{70}) phase (7). The simulation launched from random initial conditions settles into a metastable, highly defective $Fddd$ structure (Fig. 4A), whereas an analogous simulation initialized from a deterministic seed with the proper symmetry falls quickly into the stable O^{70} phase (Fig. 4B). Occasionally, large-cell simulations will also settle into defect-free, metastable phases of a different symmetry than the stable phase.

Such challenges of metastability are familiar to researchers in a variety of fields that rely on global optimization; indeed, practitioners of computational protein folding struggle with rough energy landscapes (11). Researchers in that field have the advantage that nature has apparently engineered protein sequences that are resistant to misfolding; nonetheless, they lack a quantitative mean-field theory, and their folding results do not enjoy the universality across broad families of monomers as do SCFT predictions for multiblock polymers.

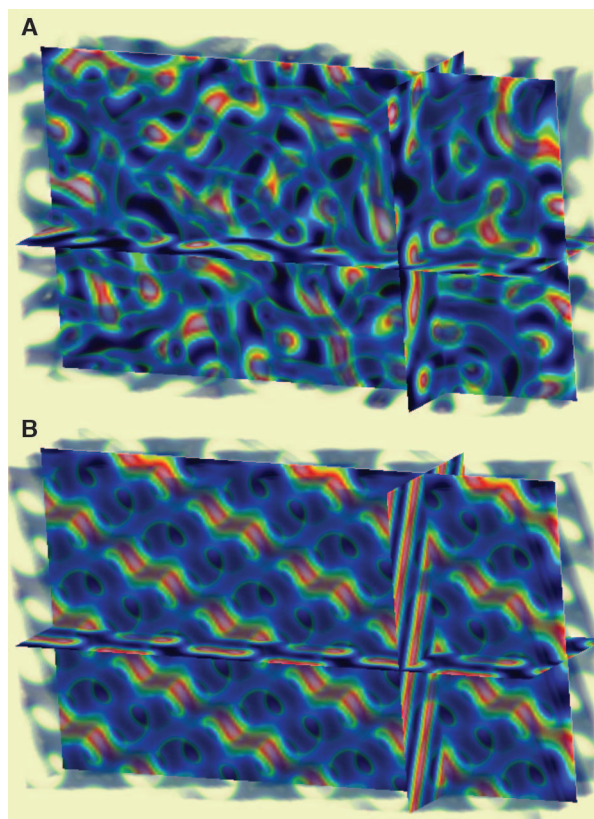


Fig. 4. Large-cell SCFT morphologies obtained for an incompressible ABC triblock terpolymer melt using parameters ($f_A = 0.275$, $f_B = 0.550$, $f_C = 0.175$, $\chi_{AB}N = 13$, $\chi_{AC}N = 35$, $\chi_{BC}N = 13$) and a periodic simulation cell size commensurate with 3-by-3-by-3 unit cells of the $Fddd$ (O^{70}) phase. (A) A highly defective structure is obtained from a simulation initialized using an unstructured, random initial field configuration. (B) A defect-free morphology comprising 27 unit cells of the O^{70} phase emerges from a simulation initialized using a small number of field harmonics consistent with the $Fddd$ space group. Both simulations were conducted on an NVIDIA Tesla C2070 graphics processing unit.

Large-cell SCFT simulations are the tool of choice when prospecting for candidate ordered phases in a new polymer system for which experimental results are not available (38). The challenges of χ_{ij} estimation notwithstanding (see Box 2), one can specify interaction parameter values, block sequences, and block lengths and then proceed to use large-cell SCFT simulations to predict microphase structure candidates. If simulations launched from a variety of random initial conditions consistently lead to a single defect-free or defective, but still identifiable morphology, one can be reasonably confident that the stable structure has been identified. Defective morphologies can also be converted to defect-free ones by the deterministic seeding method illustrated in Fig. 4. Conversely, if repeated large-cell SCFT simulations from uncorrelated random seeds turn up multiple structures differing in symmetry, then it is necessary to compare the free energies of the competitive structures to establish which of them is stable. For this purpose, a second type of SCFT simulation is most efficient—a unit-cell simulation that attempts to converge a single unit cell of a candidate structure within the confines of the symmetry constraints of that phase (41). Such a simulation seeks a saddle-point condition on the Hamiltonian with respect to the complex field variables while simultaneously minimizing H (the mean-field free energy) with respect to the lattice parameters of the unit cell.

State-of-the-art numerical methods for large-cell simulations are based on spectral collocation (or pseudospectral) techniques with plane wave bases (38, 42). Fast Fourier transforms are used to switch between real-space and reciprocal-space representations of the fields, allowing for efficient evaluation of the operators, forces, and energies embodied in SCFT. Large 3D calculations using up to $512^3 = 1.34 \times 10^8$ plane waves or grid points, such as those shown in Fig. 4, require the use of parallel algorithms implemented either on clusters of single or multicore central processing units or, more recently, on a single graphics processing unit containing up to 500 “light” cores. The method of choice for unit-cell SCFT simulations is a Galerkin spectral technique developed by Matsen and Schick that uses Fourier basis functions possessing the symmetry of the phase being considered (41). Although the method is not well suited to parallel computing, a smaller number of symmetry-adapted basis functions are generally required for accurate unit-cell simulations than the plane waves used for spectral collocation in parallelepiped cells (43).

In spite of these advances in numerical SCFT, the tool is primarily used to predict self-assembly given a block polymer design. More useful from the standpoint of applications is the inverse problem—namely, the identification of polymer designs that will self-assemble into a specified morphology. Though little progress has been made to date on this problem, techniques such as inverse Monte Carlo, in principle, could be

mated to SCFT toward this end. It is also notable that marked advances have transpired in algorithms for FTS simulations, which do not rely on the mean-field approximation (38, 44). These techniques can be used to explore a wide range of fluctuation-mediated phenomena and, when combined with thermodynamic integration and Gibbs ensemble methods, can yield accurate order-disorder phase boundaries for complex block polymers.

Outlook

Do more blocks presage a panacea or Pandora's box? At a minimum, thermoplastic elastomers require $k = 2$ and $n = 3$ as evidenced by PS-PI-PS, introduced in the 1960s and still the most successful block copolymer product in the worldwide marketplace. Increasing n at fixed $k = 2$ generates a host of new opportunities without conceptual difficulty (see Box 1) and at marginal or no added cost, provided that the appropriate synthetic tools are available. Recent advances in olefin polymerization chemistry, such as the dual-catalyst-mediated chain-shuttling mechanism (45), underscore this point. Increasing k quickly complicates the practical design of new materials yet challenges our imagination with unbounded opportunities. It is not unreasonable to claim that virtually any structure (domain morphologies and packing symmetry) can be created at length scales between roughly 5 nm and 1 μm using block polymers, while maintaining robust flexibility over individual block chemical and physical properties. Every (k, n) enumeration has the potential to produce a unique material. The resulting compounds may address engineering goals directly (e.g., multifunctional plastics) or enable a host of other products [for example, as intermediates in drug delivery, as scaffolds that guide the assembly of inorganic hard materials (46), or for pattern formation in the manufacturing of microelectronics (3)]. Realizing these tantalizing prospects requires overcoming challenges posed by complexity, a dilemma that throttles many modern technological ambitions ranging from biological systems to global climate prediction. Here, the approach

seems clear: integration of engineering goals, creative chemistry, and predictive theoretical tools, augmented by continued advances in structural characterization methods. Fortunately, even minor extrapolation in multiblock complexity (e.g., $k = 3$, $n = 4$) offers a glimpse of what is possible in the future.

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A 920-Kilometer Optical Fiber Link for Frequency Metrology at the 19th Decimal Place

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Optical clocks show unprecedented accuracy, surpassing that of previously available clock systems by more than one order of magnitude. Precise intercomparisons will enable a variety of experiments, including tests of fundamental quantum physics and cosmology and applications in geodesy and navigation. Well-established, satellite-based techniques for microwave dissemination are not adequate to compare optical clocks. Here, we present phase-stabilized distribution of an optical frequency over 920 kilometers of telecommunication fiber. We used two antiparallel fiber links to determine their fractional frequency instability (modified Allan deviation) to 5×10^{-15} in a 1-second integration time, reaching 10^{-18} in less than 1000 seconds. For long integration times τ , the deviation from the expected frequency value has been constrained to within 4×10^{-19} . The link may serve as part of a Europe-wide optical frequency dissemination network.

With residual fractional uncertainties at the 10^{-18} level (1), modern atomic frequency standards constitute extremely precise measurement devices. Besides frequency and time metrology, these standards provide valuable tools to investigate the validity of Einstein's theory of general relativity, to test

the constancy of fundamental constants, and to verify predictions of quantum electrodynamics (2–5). Furthermore, geodesy, satellite navigation, and very long baseline interferometry may benefit from steadily improving precision of both microwave and optical atomic clocks (6).

Clocks ticking at optical frequencies slice time into much finer intervals than do microwave clocks and thus provide increased resolution. Eventually, this will result in a redefinition of the second in the International System of Units (SI) (7).

Characterizing a clock requires its comparison with one or more (ideally more) precise clocks. Unfortunately, the most precise optical clocks cannot be readily transported for comparison with one another.

Hence, to link the increasing number of worldwide precision laboratories, a suitable in-

frastructure is of crucial importance. The stabilities of current satellite-based dissemination techniques that use global satellite navigation systems [such as Global Positioning System, Global Navigation Satellite System (GLONASS), and GALILEO] or two-way satellite time and frequency transfer reach a fractional uncertainty level of the frequency of 10^{-15} after 1 day of comparison (8). Whereas this is sufficient for the comparison of most microwave clock systems, the exploitation of the full potential of optical clocks requires more advanced techniques (fig. S1).

We demonstrate that optical fiber networks can provide highly accurate means for long-distance clock comparisons. Frequency dissemination via fiber networks has so far been investigated over regional distances with the use of three different methods for transmission: (i) stable microwave frequency transfer by means of an amplitude-modulated laser (9), (ii) combined transfer of radio frequency and optical signals derived from an optical frequency comb (10), and (iii) transmission of an optical carrier wave of a stable continuous wave (cw) laser system (11–13). The transmission of an optical carrier wave provides the femtosecond resolution adapted to the accuracy of optical clocks. A cw laser signal is best suited to be transmitted over large distances, as it does not require dispersion compensation and is less sensitive to polarization mode dispersion. The transmission of optical carrier frequencies via stabilized urban fiber links of up to 172 km has been shown to have residual instabilities of a few parts in 10^{16} after 1-s linear decreasing with the inverse integration time τ (14–16). The longest fiber link investigated thus far had a total length of 480 km (17). The lowest fractional uncertainty of an optical signal transmitted over 146 km has been reported to be 1×10^{-19} (15). Real-time comparison of two strontium optical clocks has been demonstrated over a distance of 120 km with the use of optical telecommunication links (18).

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Fig. 1. Map of Germany with geographic locations of all access points, distance between access points, attenuation, and signal delay along the ~920-km fiber link connecting MPQ and PTB.

Location	Length [km]	Attn. [dB]	Delay [ms]
PTB	0	0	0
Ammensleben	92	22	0.45
Cörmigk	79	21	0.39
Leipzig	114	24	0.56
Zimmritz	119	25	0.58
Großbreitenb.	79	20	0.45
Ebersdorf	74	19	0.36
Erlangen	102	23	0.50
Beilngries	88	19	0.43
Münchsmünster	82	18	0.40
MPQ	87	21	0.43
tot.	916	212	4.55



We used two 920-km-long fibers, F1 and F2, to connect the Max-Planck-Institut für Quantenoptik (MPQ) in Garching, Germany, and Physikalisch-Technische Bundesanstalt (PTB) in Braunschweig, Germany, which are separated by a geographical distance of 600 km (see Fig. 1). The fibers run in a cable conduit next to an underground gas pipeline.

Random changes of the optical path length due to acoustic noise and temperature fluctuations limit the stability and accuracy of the transmitted frequency. In our case, this results in fractional frequency excursions of $\sim 5 \times 10^{-14}$. Active noise compensation is thus a prerequisite for stable and accurate transmission. This compensation is based on actively controlling the optical frequency entering the fiber link with an acousto-optic frequency shifter (19), where the link forms the long arm of a Michelson interferometer. Details and fundamental limitations are discussed in (11, 20).

Over short distances, signal regeneration is not a critical issue and may be achieved without further in-line amplification (12, 14). In our case, the inherent attenuation of 0.23 dB/km adds to a total one-way attenuation of more than 200 dB. Hence, nine pairs of carefully adjusted Erbium-doped fiber amplifier (EDFA) systems with low noise are distributed in repeater stations over the entire link length (Fig. 1). As the phase stabilization requires identical optical paths in

both directions, all amplifiers are operated bidirectionally. In addition, we have implemented fiber Brillouin amplifiers (FBAs) with distributed gain (17) at both ends of the link, allowing coherent and fully transparent transmission. More details are given in (20).

Fiber links with the sender and receiver located in the same laboratory characterize the performance of the looped link but do not transfer a frequency between two remote users (11, 13). In this work, we used two largely identical, but independent setups in an antiparallel configuration to comprehensively characterize the frequency transfer between the two institutes (Fig. 2). From each institute, we transmit a highly stable optical frequency (194 THz) f_{PTB} and f_{MPQ} to the other institute via two fibers F1 and F2. We measure $f_{\text{F2}} = f_{\text{PTB}} - f_{\text{MPQ}} + \delta f_{\text{Laser}} - \delta f_{\text{LinkF2}}$ at PTB, and $f_{\text{F1}} = f_{\text{PTB}} - f_{\text{MPQ}} + \delta f_{\text{Laser}} + \delta f_{\text{LinkF1}}$ at MPQ and calculate $\Delta f = f_{\text{F2}} - f_{\text{F1}}$.

With this antiparallel link configuration that is equivalent to a virtual loop, the noise δf_{Laser} of both lasers cancels in the difference of the two beat notes, whereas the noise δf_{Link} of both fibers adds. Hence, we derive an upper limit for the residual instability and uncertainty of the transmitted frequency.

Due to slow drifts of the stable lasers used on both sides, any timing offset between the frequency counters at PTB and MPQ resulting

from imperfect synchronization leads to a frequency shift between the two signals $f_{\text{F1}}(t)$ and $f_{\text{F2}}(t)$. Without correction, this shift would contribute with a systematic fractional uncertainty of $\sim 1.5 \times 10^{-16}$. We determined the timing offset before each measurement by modulating one of the lasers (20). After correction, this timing offset contributes with less than three parts in 10^{19} .

We used dead-time free, high-resolution frequency counters operating in a nonaveraging (Π -type) or an averaging (Λ -type) mode (21) with a 1-s gate time to record $f_{\text{F1}}(t)$ and $f_{\text{F2}}(t)$. Fully redundant counting (22) allows us to detect possible loss of phase coherence (cycle slip) arising from a deteriorated signal-to-noise ratio. We evaluated data only for periods with no cycle slips. Under optimized conditions, the rate of cycle slips is as low as a few per day, and the signal phase can be continuously tracked for several hours. Routine operation over several days requires regular adjustment of the polarization.

In Figure 3, we show the overlapping Allan deviation (ADEV) (23) for the unstabilized and stabilized link when measured with Π -type counters. The fractional instability of $3.8 \times 10^{-14} \text{ s } \tau^{-1}$ of the stabilized link shown in Fig. 3 for a 35000-s-long data set is due to the residual broadband fiber noise. The ADEV drops off as τ^{-1} , as expected for a phase-stabilized fiber link. The observed instability agrees superbly with the theoretical

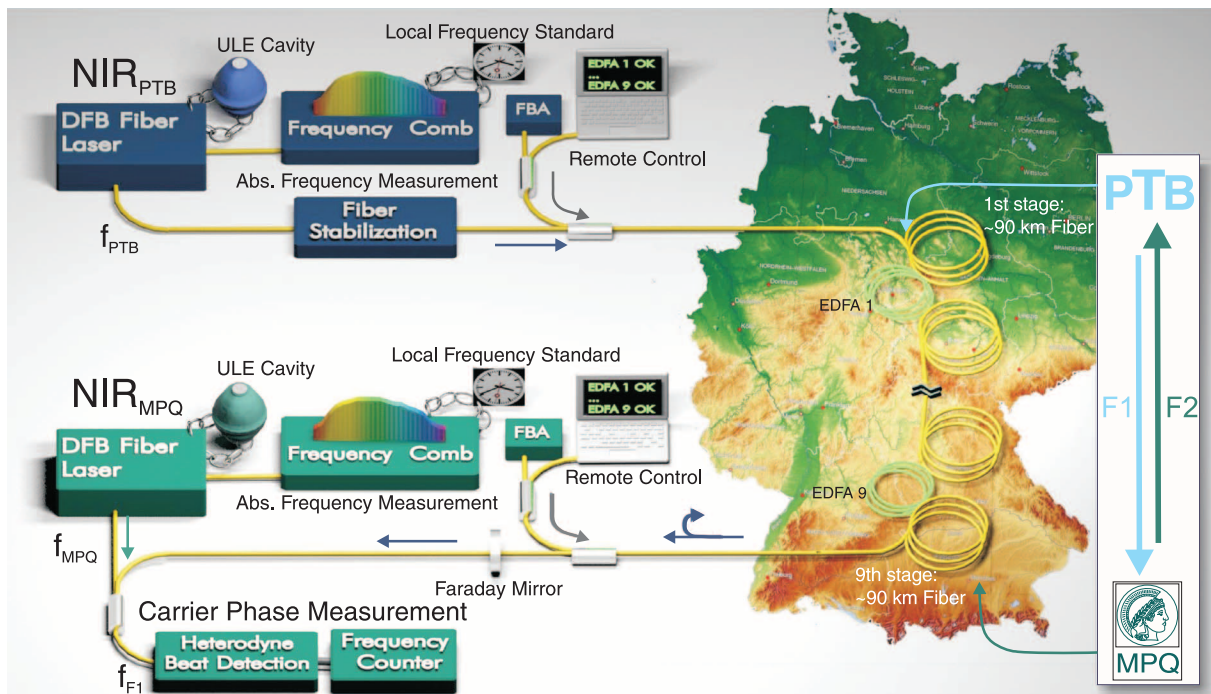


Fig. 2. Experimental setup for the characterization of two 920-km fiber links (for clarity, only one fiber is shown). Fiber F1 is used to transmit an optical frequency f_{PTB} of a distributed feedback (DFB) fiber laser (NIR_{PTB}) to MPQ. The laser operates at a frequency of 194 THz ($\sim 1542 \text{ nm}$). It shows a linewidth of a few hertz and a residual drift of $\sim 100 \text{ mHz/s}$ when locked to a high-finesse cavity made out of ultralow-expansion glass (ULE). Thus, short-term laser noise becomes negligible compared with the phase noise of the free-running fiber link (19). By means of a femto-

second comb (25), the laser's frequency is measured against the local microwave standard. F1 is actively stabilized in a single step and further equipped with 11 optical amplifier systems (remotely controlled EDFAs and FBAs) to preserve the signal power and coherence. To avoid stimulated Brillouin scattering, we limit the power launched into the fibers to 6 mW. At MPQ, a heterodyne beat note f_{F1} between f_{MPQ} and f_{PTB} is measured. Likewise, we transmit f_{MPQ} via fiber F2 from MPQ to PTB and generate a beat note f_{F2} .

prediction in (15), thus providing strong confirmation of the $L^{3/2}$ scaling law derived in (11), for distances up to 1000 km.

To demonstrate the link's instability floor and illustrate the potential of adapting the type of averaging to the dominant noise type, we addition-

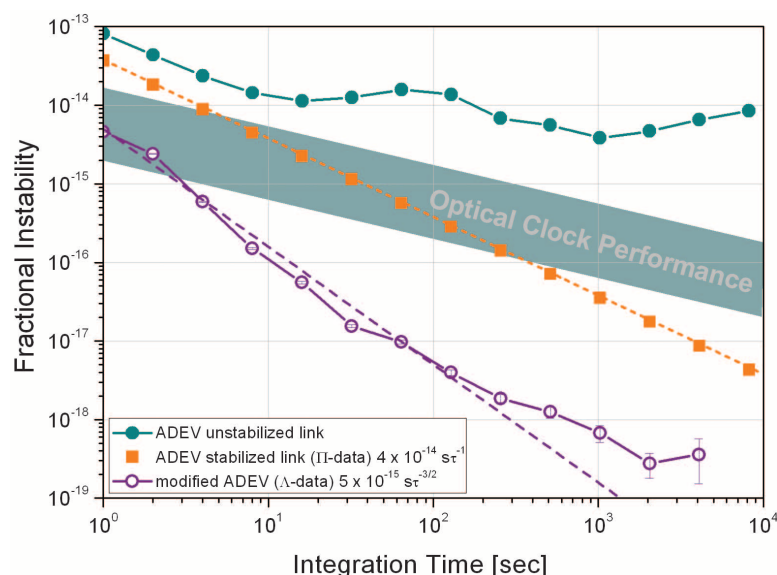


Fig. 3. Fractional instability of two 920-km telecom fiber links F1 and F2 connecting PTB and MPQ. We show the overlapping ADEV of the unstabilized link (solid circles) and stabilized link (squares) derived from a nonaveraging counter for a 35000-s-long data set. We also show the modified ADEV (open circles) for a 18000-s-long data set from data taken with averaging (Λ -type) counters, which allows us to distinguish white phase noise from flicker phase noise (21). The ADEV for state-of-the-art optical clock performances is depicted for comparison. The dashed lines indicate the expected stability curves for a signal dominated by white phase noise.

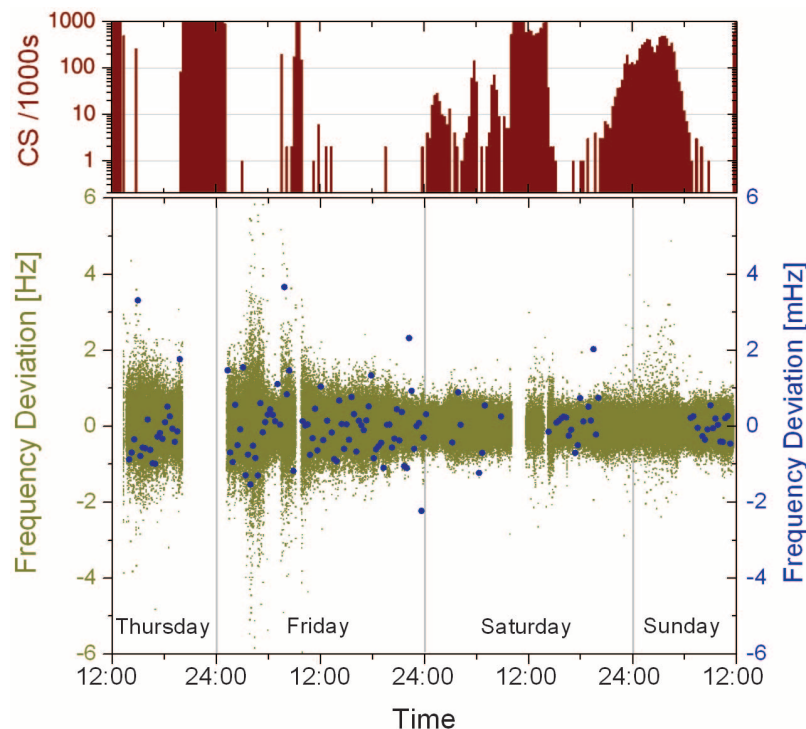


Fig. 4. Four-day frequency comparison between MPQ and PTB. Data are taken with high-resolution Λ -type frequency counters (overlapping triangle weighting, no dead time) with 1-s gate time (green data points, left frequency axis). Cycle slip (CS) rates per 1000 s are shown in the upper plot. Phase-coherent intervals have been binned into 1000-s-long sequences and averaged. From the 135 resulting data points (dark blue, right frequency axis, enlarged scale), we calculated a fractional deviation from the expected frequency of $(0.7 \pm 3.7) \times 10^{-19}$.

ally show data, where the counters were operated in Λ mode (overlapping weighted average). As shown in Fig. 3 for a 18000-s-long data set, we achieve a residual instability (modified ADEV) of 5×10^{-15} in 1 s. It averages as $\tau^{-3/2}$ for up to 100 s and reaches 10^{-18} in less than 15 min. The deviation of the fractional instability from the $\tau^{-3/2}$ slope for $\tau > 300$ s is mainly due to a few tens of centimeters of uncompensated fiber in the MPQ laboratory that have not been temperature-stabilized. We estimated the corresponding uncertainty for the MPQ interferometer to be $< 3 \times 10^{-19}$ (20).

Figure 3 demonstrates that the fiber-link instability does not contribute substantially to the uncertainty budget of a frequency comparison when comparing even the world's most stable optical clocks (1) over a distance of 920 km.

The accuracy of the transmitted signal might be compromised by systematic offsets that would not be observed in the instability analysis. From data taken with a Λ -type counter over a period of 4 days, we selected all continuous data subsets with a length of 1000 s, as shown in Fig. 4. The arithmetic mean of each subset is calculated to benefit from the strong suppression of white phase noise for longer integration times. The resulting 135 data points have a mean of 13 μHz and a SD $\sigma = 823 \mu\text{Hz}$; more than 80% of the values are within 1σ (fig. S2). The statistical fractional uncertainty of the mean is 3.7×10^{-19} . Thus, we can constrain the deviations from the expected frequency value to be less than 4×10^{-19} . This value exceeds the requirements posed by the most accurate atomic clocks to date by more than one order of magnitude.

As a first application, we have used the fiber link to measure the 1S-2S two-photon transition frequency in atomic hydrogen at MPQ, using PTB's primary Cs-fountain clock (CSF1) as a reference (24). The hydrogen experiment constitutes a very accurate test bed for quantum electrodynamics and has been performed at MPQ with ever increasing accuracy. The latest measurement (3) has reached a level of precision at which satellite-based referencing to a remote primary clock would limit the experiment. As the transmission over fiber is up to three orders of magnitude more stable, a frequency measurement can be carried out straightforwardly without the need for transporting a frequency standard to the experiment.

The link now permanently connects MPQ and PTB and is operated routinely. Going beyond a proof-of-principle experiment conducted under optimized laboratory conditions, it constitutes a solution for the topical issue of remote optical-clock comparison, opening a variety of applications in fundamental physics, such as tests of general and special relativity as well as quantum electrodynamics. Furthermore, such a link will enable clock-based, relativistic geodesy at the subdecimeter level. Applications in navigation, geology, dynamic ocean topography, and seismology are currently being discussed. Some of

the future applications of optical clocks will be space-based, and their operation will require ground stations with access to ultrastable frequency references linked to the best available clocks. In this respect, optical links will become of central importance.

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Revealing the Angular Symmetry of Chemical Bonds by Atomic Force Microscopy

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We have measured the angular dependence of chemical bonding forces between a carbon monoxide molecule that is adsorbed to a copper surface and the terminal atom of the metallic tip of a combined scanning tunneling microscope and atomic force microscope. We provide tomographic maps of force and current as a function of distance that revealed the emergence of strongly directional chemical bonds as tip and sample approach. The force maps show pronounced single, dual, or triple minima depending on the orientation of the tip atom, whereas tunneling current maps showed a single minimum for all three tip conditions. We introduce an angular dependent model for the bonding energy that maps the observed experimental data for all observed orientations and distances.

When more than two atoms are involved, the forces that act between atoms generally depend not only on their distance but also on the angles between the atoms. Chemical bonds establish an equilibrium between attractive and repulsive interactions for atoms that is described by the Morse potential (1). When more than two atoms are involved, the bonding energy generally depends on the bonding angle as well as distance, and model potentials with an angular dependence, such as the Stillinger-Weber potential (2), are needed. The directional character of the atomic bonds is reflected in the crystal structure. Most metals condense in a face-centered cubic (fcc), hexagonal close-packed (hcp), or body-centered cubic (bcc) lattice (3). In particular, metals with unfilled p or d shells can show strong directional bonding [chapters 1.4 and 2.5 in (3)]. Although fcc and hcp lattices have the largest possible

number of nearest neighbors, the angular dependence of atomic forces leads to slight energetic differences between fcc and hcp lattice structures. Atomic manipulation of cobalt atoms on a copper surface by scanning tunneling microscopy (STM) has shown that a Co adatom on a Cu(111) surface prefers to occupy an fcc site over an hcp site, although the adatom is in both cases sitting directly in a dip between three Cu atoms (4).

Atomic force microscopy (AFM) (5) can measure forces between individual atoms with great precision, and recent progress in three-dimensional force spectroscopy (6–8) helps to investigate the angular dependence of atomic bonding forces. Initial manifestations of an angular dependence of bonding forces led to the observation of subatomic features on the surface of Si (9). The data were explained by the directional dependence of covalent bonds between a Si tip with a Si adatom on the sample [figure 4 in (9)] as predicted by calculating the tip-sample interaction using the Stillinger-Weber potential (2), a classic model potential for diamond structure materials. Density functional theory (DFT)

calculations by two groups (10, 11) supported the covalent-bonding theory, but other authors (12) proposed that the data could have been the result of a feedback artifact, and there has been a recent theoretical proposal that the angular dependence could be caused by multiple-atom tips (13). Simultaneous STM and AFM experiments of a W tip imaging a graphite sample showed a single current maximum and multiple force maxima in the image of the W tip atom (14); hence, AFM can address electronic states that are spatially more confined than electronic states that are accessible to STM. In that experiment, the light and small carbon atoms have produced repeated images of the electronic valence states in tungsten atoms [see figures B and C in (15)]. Recent DFT calculations confirmed that the electronic structure of W should show these small features (16). However, the softness and close atomic packing of graphite make it difficult to calculate the experimental observables, and distance-dependent data were not available in the first experiments.

Precise measurements of the interaction of two single and clearly defined atomic bonding partners as a function of distance and angle would allow us to study the evolution of the atomic angular dependencies and provide well-defined experimental data that can serve as a reference for theoretical calculations. Here, we present an experimental study of the interaction of two well-defined bonding partners: a single CO molecule that is bonded to a Cu(111) surface and the metallic front atom of a tip. We analyzed tunneling current and frequency shift in three dimensions and were therefore able to recover the force and potential energy between two atomic bonding partners as a function of angle and distance.

We used STM (17) combined with frequency modulation AFM (18), where a quartz cantilever (qPlus sensor) (19) with a stiffness of $k = 1800$ N/m, an eigenfrequency of $f_0 = 27275.20$ Hz, a quality factor of $Q = 195870$, and a W tip oscillated with a constant amplitude A (here, we

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use $A = 50$ pm and $A = 100$ pm). When the tip of the cantilever was subjected to a tip-sample force F_z with gradient $k_z = -dF_z/dz$, the frequency shifted to $f = f_0 + \Delta f$ with

$$\Delta f = f_0 < k_z > / (2k) \quad (1)$$

Our commercial cryogenic scanning probe microscope, operated at 4.3 K (20), measured an average tunneling current $\langle I \rangle$ in parallel with an average force gradient $\langle k_z \rangle$ (21). The interaction between tip and molecule was probed by three-dimensional force spectroscopy (6–8), where a set of constant height images spaced by a vertical distance of 10 pm was collected to recover the energy profile. We used the method of Sader and Jarvis (22) to recover forces and energies from frequency shifts (23) (figs. S1 and S2). Because

of the small oscillation amplitudes we used, the frequency shift was approximately proportional to the force gradient, so that the atomic short-range contributions (ranging from a few piconewtons up to ≈ 300 pN) could be measured with a high signal-to-noise ratio on top of the long-range van der Waals forces ranging up to 2 nN (fig. S3).

The tips were made from a multicrystalline W wire and cleaned and prepared in situ by electron bombardment and field evaporation at voltages up to 15 kV. Because the tips were prepared and transferred into the low-temperature microscope in an ultrahigh vacuum, we assumed that they expose clean surfaces with a W front atom.

Carbon monoxide is known to chemisorb via its C atom onto a site on top of a Cu atom on the Cu(111) surface (24). It can be manip-

ulated in a controlled fashion on the Cu(111) surface and is observed as a dip in low-bias STM experiments (25). Figure 1 shows the setup of the experiment: A W tip was placed above a CO molecule that is chemisorbed on a Cu(111) surface. Three highly symmetric tip configurations are studied in detail here: Fig. 1A depicts tip 1, a W tip presumably oriented in a [001] direction. This tip was poked into the Cu(111) surface to change its geometry. This tip-shaping procedure used is standard in the low-temperature STM community, and it is commonly assumed that poking the tip in Cu is likely to cover the tip apex with Cu [e.g., (25, 26)]. Further below, we provide evidence that, although tip 1 was poked into the Cu surface, it had a W atom at its front. Figure 1E shows tip 2, a W tip presumably oriented in a [011] direction with a twofold

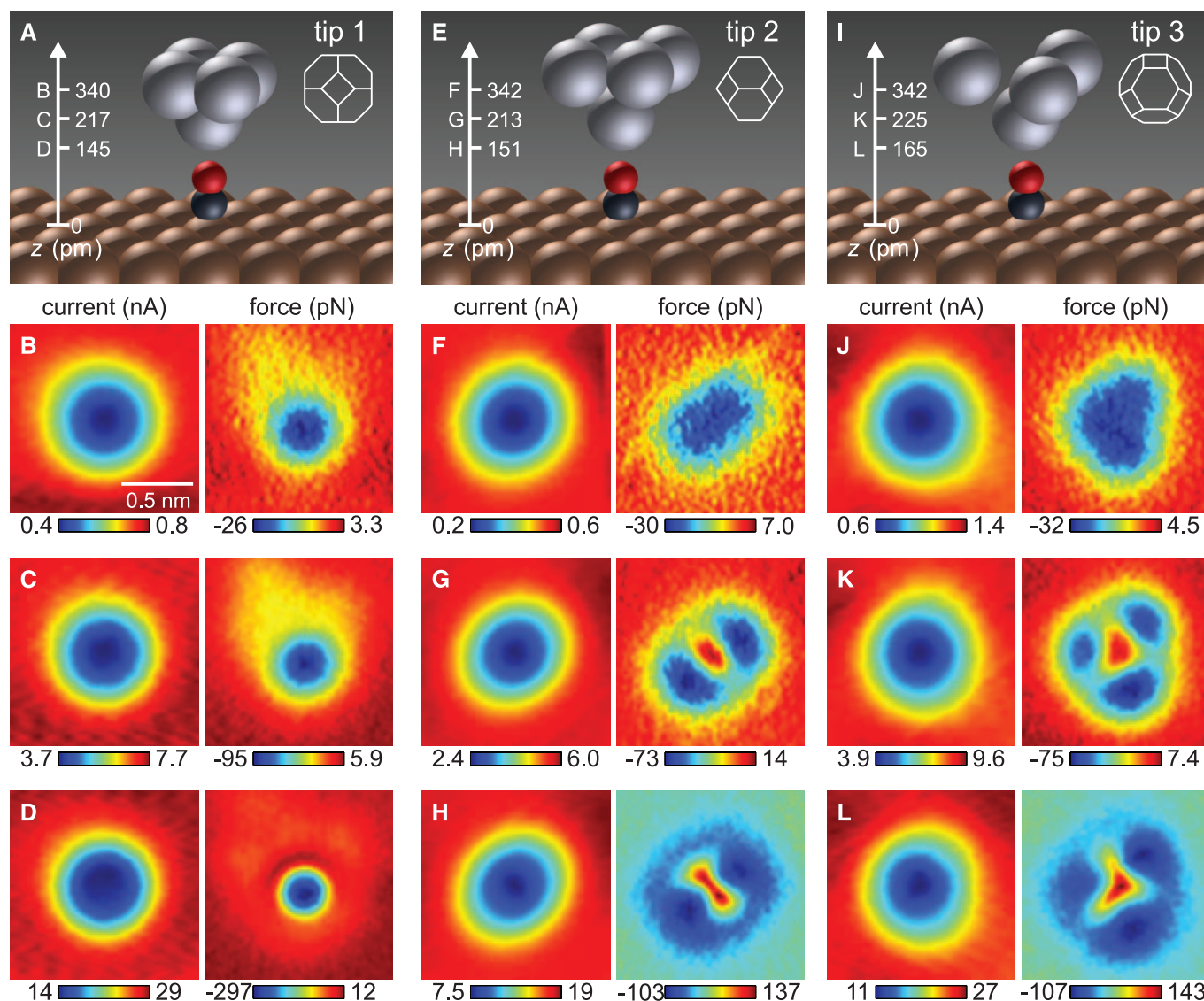


Fig. 1. Constant height images of tunneling current and deconvoluted force for three different tips over a CO molecule on Cu(111) recorded at three different heights (see axis indicators in top row): (A to D) current and force for a tip that is highly symmetric with respect to rotations around the z axis; (E to H) current and force for a tip with a twofold dip in the force; (I to L) current and force for a

tip with a threefold dip in the force; sample bias is +10 mV, and cantilever amplitudes are $A = 50$ pm (tips 1 and 3) and 100 pm (tip 2). The tunneling current follows an exponential law with $I(z) = I_{\max} \exp(-z/\lambda_t)$, where $\lambda_t = 55$ pm, 56 pm, and 59 pm for tips 1, 2, and 3, respectively. The forces refer to the atomic contributions; long-range interactions are not included here.

rotational symmetry with respect to the z axis that causes a double dip in the force maps. Tips 2 and 3, however, were treated with great caution after the in situ high-voltage preparation to maintain a safe distance to the sample such that we are sure they expose clean W at the apex. Figure 1I depicts tip 3, a W tip presumably oriented in a $[111]$ direction with a threefold rotational symmetry with respect to the z axis causing a triple force minimum. The transition from tip 2 to tip 3 occurred after a gentle "poke" (a voltage pulse that caused, among structural changes in the tip apex, single W atoms to drop from the tip onto the Cu surface). As can be seen in fig. S3, B and C, the tip radius was not affected by that transition.

A gentle poke event was conducted in the following way: First, the tip was held at a tip height (27) over the Cu surface of about 400 pm at a fixed lateral position. Then, the tip approached the surface by about 700 pm to the surface, so it was driven by about one atomic diameter into the surface. After that, a bias voltage of a few volts was applied, and the tip was slowly retracted within a few seconds. This procedure led to structural changes in the tip apex, and often to dropping one or a few tip atoms onto the Cu surface, whereas the Cu surface remained atomically flat otherwise. This finding is somewhat surprising, because W is much harder than Cu, and one might expect to find depressions in the flat Cu surface after a poke. However, even at the Cu(111) surface layer, a Cu atom still has 9 nearest neighbors (out of 12 in bulk), whereas W atoms at the tip apex have only a fraction of the eight nearest neighbors present in bulk W. (More information about the statistics of poking events is provided in fig. S10.)

The data sets in rows 2 to 4 in Fig. 1 are tomographic slices of current and force (short-range contribution only) for three different heights, starting with a height (27) of about 340 pm for the top row starting with Fig. 1B, about 220 pm for the center row starting with Fig. 1C, and about 150 pm for the bottom row starting with Fig. 1D (for precise values, see the marks on the z axes in Fig. 1, A, E, and I). At least 40 slices of current and frequency shift, spaced by height decrements of 10 pm, have been probed for each of the tips 1 to 3, and movies S1 to S3 show 40 frames starting at $z_{\text{max}} \approx 550$ pm and ending at $z_{\text{min}} \approx 150$ pm. Evidently, the force signals show a striking angular dependence that becomes more pronounced with decreasing tip height z .

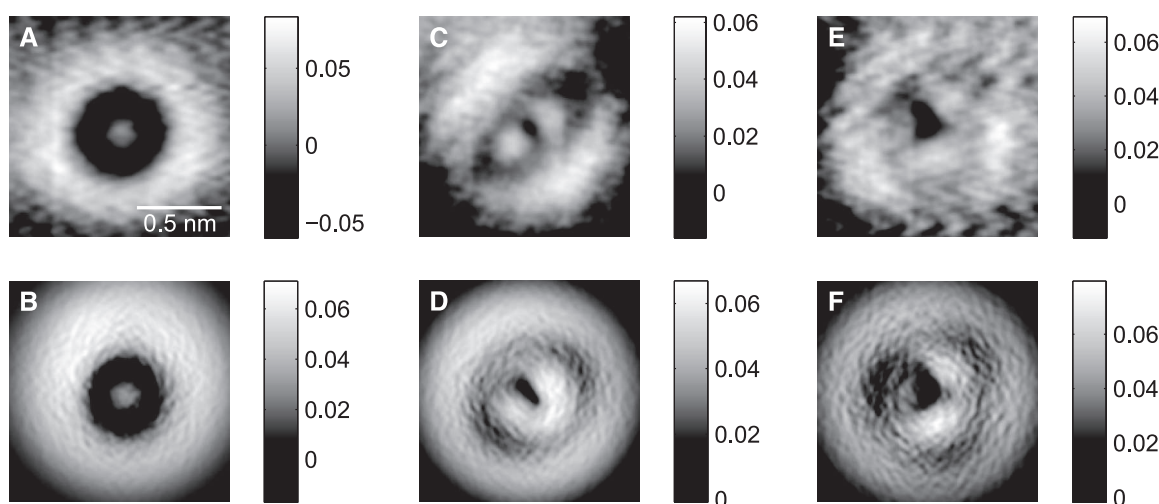
This pronounced angular dependence of the frequency shift (and force) data, showing dual and triple minima for tips 2 and 3, respectively, is in stark contrast to the z -rotational symmetric force profiles that were found for metallic adatoms and metallic tips (28), presumably Cu-covered Ir tips and CO/Cu(111) (7), and a CO-terminated metal tip interacting with CO/Cu(111) (29). At a first glance, the current images in Fig. 1 and in movies S2 and S3 appear to be symmetric with respect to rotations around the z axis. Binnig suggested that it is possible to search for a potential angular current dependence by subtracting the normalized current data at a large distance from the normalized current data near the surface. The top row of Fig. 2 shows these differential normalized current images for tips 1, 2, and 3. Indeed, the differential images do show an angular dependence that reflects the rotational symmetry of the force images, although the local extrema are closer to the center in the differential current images than in the force images. Thus, some of the angular dependence of the

force data also seems to show up directly in the tunneling current.

An influence of atomic forces on current data has been observed with giant corrugations on soft samples (30) and corrugation enhancements on metal surfaces (31). The force field between an STM tip and a molecule can trap hydrogen molecules that modulate the current to improve molecular resolution (32). Although the free CO molecule stands perfectly upright on top of a Cu surface atom, it can be deflected laterally by external forces (29). The softest vibrational mode of CO on Cu(111) is the frustrated translation mode, with an energy of 4 meV as measured by He scattering (33) and by inelastic tunneling spectroscopy (26). The vibrational energy and the molecule's moment of inertia lead to an estimate of the lateral force constant k_{CO} (figs. S4 and S5). The lateral forces between tip and CO molecule were obtained from the experimental data by differentiating the energy profile with respect to a lateral direction within the (x, y) plane (fig. S6). For each position (x, y) , we calculated the tilt of the CO molecule by dividing the lateral force by k_{CO} . The tunneling current as a function of (x, y) at constant height could be simulated with a Gaussian dip (see captions of Fig. 2 and fig. S5). Figure 2, B, D, and F, shows the simulated current images with $k_{\text{CO}} = 2.1$ N/m. The simulated images provide a very good match to the experimental images, demonstrating that the apparent angular dependence in the current images is likely caused by a position-dependent tilt of the CO molecule that modulates the current rather than a direct angular dependence of the tunneling current.

Next, we ask what we can learn about the physics of the bond from the experimental spatial force dependence. Experimentally, we have

Fig. 2. Experimental and simulated differential images of the tunneling current. The tunneling current above a CO molecule at position (x_0, y_0) can be approximated well by a Gaussian dip $I(x, y) = I_0(1 - \Delta \exp\{-(x - x_0)^2 + (y - y_0)^2/\rho^2\})$, where ρ was fitted to the experimental current profiles at large distance, yielding $\rho = 387$ pm, 401 pm, and 431 pm for tips 1, 2, and 3, respectively, at $z \approx 350$ pm. ρ is about 9% smaller for $z \approx 150$ pm. The normalized tunneling current is obtained by setting the maximum current (above Cu) to 1 and the minimum (at the center of CO) to 0. The experimental difference between the normalized tunneling current close to the surface ($z \approx 150$ pm) and far from the surface ($z \approx 350$ pm) is shown in (A), (C), and (E) for tips 1, 2, and 3. The simulated difference between the normalized tunneling current



close to the surface ($z \approx 150$ pm) and far from the surface ($z \approx 350$ pm) shown in (B), (D), and (F) is calculated by assuming a Gaussian dip for the current distribution but allowing a lateral tilt of the CO molecule $(x', y') = (F_x/k_{\text{CO}}, F_y/k_{\text{CO}})$ caused by the experimentally determined lateral forces (figs. S4 and S5).

access to the net force F in z direction. The semi-empirical Morse potential (I)

$$V_M(d) = E_{\text{bond}}[-2e^{-(d-\sigma)/\lambda} + e^{-2(d-\sigma)/\lambda}] \quad (2)$$

describes the bonding energy of a diatomic molecule as a function of the atomic distance d . The two terms describe attractive and repulsive interaction components for two atoms with a decay length λ for the attractive and $\lambda/2$ for the repulsive branch of the potential, reaching its minimum (bonding energy) at the equilibrium distance σ .

If the force between tip and sample could be fully described by a Morse potential, the force images would show a single attractive minimum with a similar lateral extension as the dip in the current image, and the force images would be symmetric with respect to rotations around the z axis. For tip 1, the force data were approximately symmetric with respect to rotations around the z axis, but the force minimum was laterally markedly more confined (diameter ≈ 330 pm in Fig. 1 D) than the current minimum (diameter ≈ 720 pm). Tips 2 and 3 showed a strong azimuthal dependence in the force image. For tip 2, we found two local minima that became more pronounced with decreasing distance (Fig. 1, F to H). Tip 3 exposed three minima that also became more pronounced with decreasing distance.

Figure 3 shows experimental force versus distance data for tips 1 (panel A), 2 (C), and 3 (E).

Tip 1 showed an attractive force that became more attractive with decreasing distance and reached its greatest magnitude at a distance of 145 pm. A closer approach to the surface would have caused a lateral force greater than the moving threshold of 160 pN (7) and would have pulled the CO molecule laterally. In the distance regime from 500 to 200 pm, the force increased exponentially in magnitude with decreasing distance with a decay length of 95 pm. For distances < 200 pm, the attraction increased faster at a decay length of 40 pm.

Figure 3C refers to tip 2, where the force curve at the center (red) could be well approximated by a Morse law with $E_{\text{bond}} = 5.95$ zJ, $\sigma = 217$ pm, and $\lambda = 93$ pm. At the lateral positions where attraction was strongest (blue graph in Fig. 3C), we did not get close enough to the surface to find an absolute force minimum. The difference between the force curves at the center (red) and the most attractive sites (blue) was well approximated by an exponential function with a decay length of 40 pm.

Figure 3E shows the force-versus-distance dependence of tip 3, where the force curve at the center (red) was well approximated by a Morse law with $E_{\text{bond}} = 6.30$ zJ, $\sigma = 222$ pm, and $\lambda = 99$ pm. The blue curve in Fig. 3E shows the force at the lower one of the three attractive lobes. Again, this curve did not show a minimum within the distance range that was covered, and the

difference of the center (red) and attractive lobe curves (blue) could be approximated by an exponential function with a decay length of 40 pm.

The dependence of the force curves as a function of crystal direction and distance allowed us to build a semi-empirical potential $V_{\text{W-CO/Cu(111)}}$, which was composed of a Morse potential and a very short-range, additional attractive component pointing in $<100>$ directions that accounts for the three tip symmetries shown in Fig. 1 as well as the lower-symmetry tips shown in fig. S9. We assumed that the force symmetry was related to the crystal symmetry of the tip material. The crystal structure of W is bcc, and Fig. 3G shows the Wigner-Seitz cell of bcc W. The bcc Wigner-Seitz cell is fourfold symmetric in $<001>$ directions, twofold symmetric in $<011>$ directions, and threefold symmetric in $<111>$ directions. If we assume that the angular dependence is caused by a bonding energy component that is strong along the $<001>$ directions, we can explain the data in Fig. 1 for all three tip orientations. A mathematical function that reflects this angular symmetry is given by

$$\alpha_n(x'/r', y'/r', z'/r') = 1/(1 - 3^{1-n})[(x'/r')^{2n} + (y'/r')^{2n} + (z'/r')^{2n} - 3^{1-n}] \quad (3)$$

with $r' = (x'^2 + y'^2 + z'^2)^{0.5}$ and an integer $n \geq 2$ (fig. S7). This function is constructed to yield $\alpha_n = 1$ for the $<100>$ directions, $\alpha_n = 0$ for all

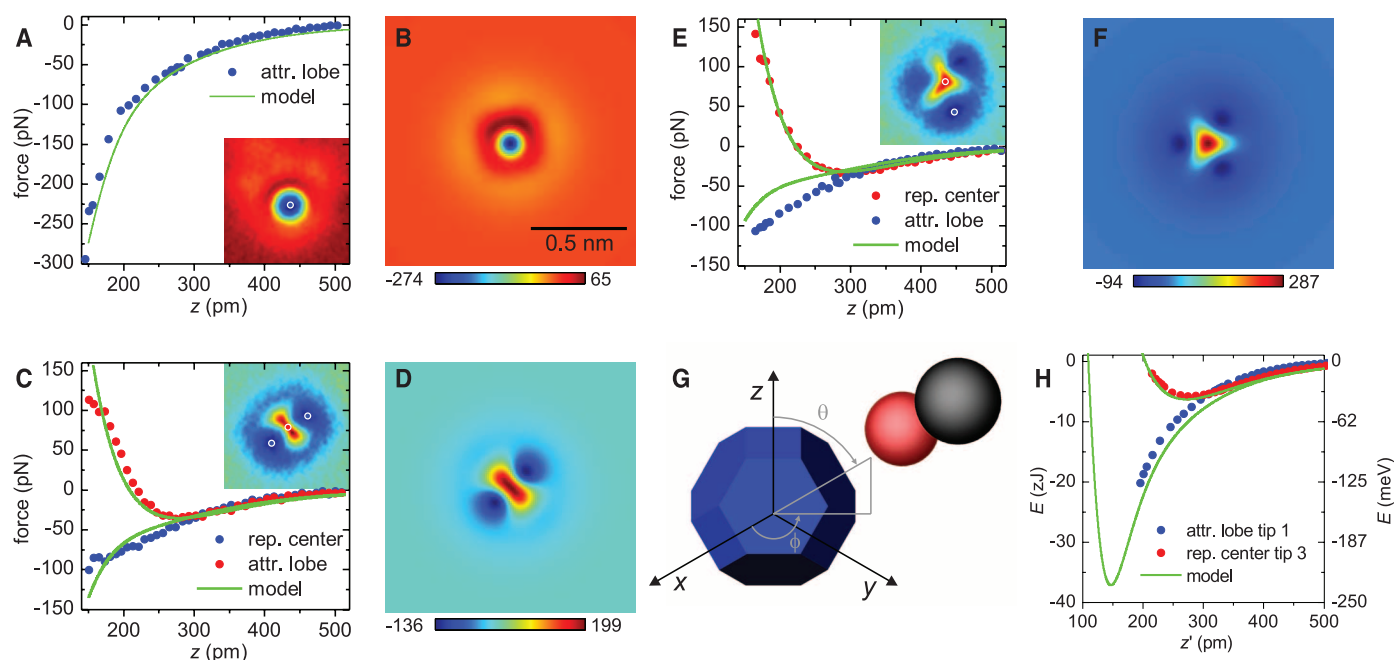


Fig. 3. Force versus distance data and fit curves for tip 1 (A), tip 2 (C), and tip 3 (E). For tips 2 and 3, the force curve is fitted at the local maxima (red) and minima (blue); see insets. The fit curves are composed from a Morse potential and an angular dependent attractive exponential term. (G) Representation of the W tip atom as its Wigner-Seitz unit cell and orientation of a CO molecule with azimuthal angle ϕ and inclination angle θ . Here, the $[111]$ orientation is shown with $\theta = \arccos(1/\sqrt{3}) \approx 54.73^\circ$ and $\phi = 45^\circ$. The $[110]$ orientation corresponds to $\theta = 45^\circ$ and $\phi = 0^\circ$, and the $[100]$ orientation is obtained for $\theta = 0^\circ$ and $\phi = 0^\circ$. For clarity, only the front atom

of the tip and the CO molecule are shown. (B) Force derived from the model potential at a height of 150 pm and a tip orientation close to $[001]$ with $\theta = 5^\circ$ and $\phi = 10^\circ$. (D) Force derived from the model potential at a height of 150 pm and a $[110]$ tip orientation with $\theta = 45^\circ$ and $\phi = 0^\circ$. (F) Force derived from the model potential at a height of 150 pm and a $[111]$ tip orientation with $\theta \approx 54.73^\circ$ and $\phi = 0^\circ$. (H) Bonding energy as a function of distance for tips 1 and 3 showing experimental data and the results from our model. Note that the horizontal scales in (A), (C), and (E) and z and z' in (H) are shifted by 50 pm (fig. S7).

<111> directions, and $\alpha_n = (1.5^{n-1} - 1)/(3^{n-1} - 1)$ for the <110> directions (e.g., $\alpha_2 = 1/4$, $\alpha_3 = 5/64$). Because the angular dependent force contribution shows an approximately exponential decay for larger distances (difference of red and blue curves in Fig. 3, C and E), we model the full angular potential by

$$V_{W-CO/Cu(111)}(x', y', z) = V_M(r') - \alpha_n(x'/r', y'/r', z'/r') \times E_{ang} \exp[\sigma_{ang}/\lambda_{ang} - (r'^8 + \sigma_{ang}^8)^{1/8}/\lambda_{ang}] \quad (4)$$

This potential has seven parameters, and a good agreement between the experimental force versus distance data and constant height images is obtained for $E_{bond} = 6.30$ zJ, $E_{ang} = 110$ zJ, $\sigma = 272$ pm, $\lambda = 99$ pm, $\sigma_{ang} = 140$ pm, $\lambda_{ang} = 40$ pm, and $n = 3$. The green lines in Fig. 3, A, C, and E, show the forces as obtained from Eq. 4. Figure 3, B, D, and F, shows the calculated constant height force images obtained by using the model potential. The key features and the signs and magnitudes of the experimental forces are accurately generated by that potential. Even subtle features in $V_{W-CO/Cu(111)}$, such as the appearance of a small repulsive ring around the attractive center of Fig. 3B, were observed in the experiment. The minima are spaced wider apart in the experimental data than in the calculation according to the model potential $V_{W-CO/Cu(111)}$, possibly because of the lateral bending of the CO molecule discussed in Fig. 2.

The design of our model potential after Eq. 3 is supported by the physics of chemical bonding. The Morse part of $V_{W-CO/Cu(111)}$ is treated in most textbooks on quantum mechanics by calculating, for example, the bonding energy of the hydrogen molecular ion by perturbation theory, where the Morse potential is an approximation for the energy as a function of distance for the symmetric pair wave function.

The angular dependent part is more complicated. In his paper (34), Feynman demonstrated that forces in molecules (and thus in tip-sample interactions) can be calculated from electrostatics once the wave functions have been found by solving Schrödinger's equation. Because the valence electrons of CO form lone pairs, we do not expect that CO develops a strong electronic overlap with the W tip atom. In bulk W, the atoms form bonds to their next neighbors across the hexagons in Fig. 3G that point toward the corners of the cubic unit cells in the eight <111> directions. Even for W atoms at the surface, calculations show that the valence charge density has maxima in the <111> directions (16, 35, 36), so each W atom can be viewed as an electric multipole with a positively charged center and eight negative charges pointing in the <111> directions (fig. S8). Because the lone pairs in CO cannot form molecular orbitals with the W electrons, we modeled the interaction by an electrostatic dipole of the CO molecule interacting with the multipoles of the W atoms. If the CO dipole has a negative charge on the oxygen atom, attraction occurs along the <100> directions (fig.

S8) and repulsion along the <111> directions. The exponential decay of this strongly directional force is also motivated by electrostatics. The electrostatic potential at the surface of a periodic array of charges with lattice constant a decays proportional to $\exp(-2\pi z/a)$ (37). The lattice constant of W is 273 pm, and the corresponding decay length is, at $273 \text{ pm}/2\pi = 43$ pm, very close to our fit parameter, $\lambda_{ang} = 40$ pm.

The precise measurement of forces and currents between a metallic tip and CO/Cu(111) allows us to identify the tip. Initially, we assumed that tip 1 in Fig. 1A was covered by Cu because it has been prepared by poking into the Cu surface. To check this assumption, we prepared a Cu surface with a Cu adatom and picked up a CO molecule with the tip; if the short-range force depends only on the bonding partners, the force spectrum should be the same for a CO-terminated tip probing a Cu adatom as for a Cu-covered tip probing a CO molecule adsorbed on Cu. Experimentally, the attractive force between a CO-terminated tip and a Cu adatom was below 30 pN, only one-tenth of the attraction of tip 1 with CO. Our model potential $V_{W-CO/Cu(111)}$ explains the magnitudes of forces and the geometric details of the force with the same parameters as for tips 2 and 3 very well for a [001] orientation. Thus, we are confident that tips 1, 2, and 3 are all W front atom tips oriented in [001], [011], and [111] directions, respectively. Our model potential from Eq. 4 even provides a very good agreement with experimental images recorded with tips that are oriented at odd angles—that is, tips that are not aligned with high-symmetry crystal directions (as shown in fig. S9).

Tip 1 exerted an attractive force of 300 pN at a distance of 150 pm, and this tip worked reasonably well for atomic manipulation. Lateral forces are known to be relevant in atomic manipulation (7), and the lateral force field that was generated from tip 1 (fig. S4A) allowed reliable lateral dragging of adsorbed CO molecules (or other adatoms) across the surface. Tips 2 and 3 did not provide enough attractive force for reliable manipulation of CO and tended to push adsorbed CO away from the tip in a less-controlled fashion (fig. S4, A and B). Probing the tips with a CO molecule allowed us to assess the effectiveness of a given tip for atomic manipulation and provided immediate feedback about the success of a tip-poking event to form a suitable tip (see fig. S10 for a histogram of obtaining tips of type 1 to 3 by poking). We note that figures 1D and 3A in (7) showed a force profile with a slightly repulsive crescent around the wide attractive valley, similar to our tip 1.

Lastly, we discuss the origin of the difference of appearance in force data versus tunneling current data. Stoll (38) has discussed how the magnitude of the decay length of the tunneling current connects to spatial resolution and found that the smaller the spatial decay length, the greater the spatial resolution can become (fig. S11). The angular part of the interaction potential

has a decay length of only about 40 pm, whereas the decay length of the tunneling current ranges from 53 to 60 pm. Probing the short-range interactions at very small distance also increased spatial resolution from molecular resolution in the attractive regime to atomic resolution in the repulsive regime when imaging pentacene by Gross *et al.* (39). A density-functional study (40) has found that the origin of the submolecular contrast is Pauli repulsion with a very small decay length.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/336/6080/444/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S11
References (43, 44)
Movies S1 to S3

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Coils and Polygonal Crust in the Athabasca Valles Region, Mars, as Evidence for a Volcanic History

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Athabasca Valles is a near-equatorial martian outflow channel that contains many well-preserved features whose formation and composition have been a point of contention. Large plates of terrain that have clearly fractured and drifted may have once been ice rafts or the rocky solidification crust of a large lava flow. We have identified 269 spiral coils ranging from 5 to 30 meters wide on the polygonally patterned interplate terrain that are morphologically consistent with terrestrial lava coils that form in zones of flow shear. This patterned terrain also exhibits signs of fracture and drift, indicating that it is platelike as well. The coils in the Athabasca region are inconsistent with ice rheology, and the plates, spirals, and polygons are interpreted to be of volcanic origin.

Athabasca Valles contains some of the best-preserved volcanic and erosional features on Mars. Cerberus Palus, a plain southwest of Athabasca, contains many large fractured plates of material that show clear signs of break-up and horizontal drift over distances of several km. Some authors have likened these plates to pack ice (1) while noting that other local features, such as patterned ground, are evidence of recent and potentially ongoing modification by persistent near-surface ice (2–4). Conversely, others have concluded that the region is entirely mantled by lava that erupted turbulently from one of the southwestern-most fissures of the Cerberus Fossae (5–9). Although pack ice and lava rafts share similar rheologies and can produce similar forms, a combination of regional context and finer-scale morphologic and textural characteristics, including the features described here, can be used to definitively differentiate between these alternate interpretations.

Crater count densities indicate that resurfacing occurred in the Athabasca Valles region within the past 200 million years, suggesting that it is one of Mars' youngest outflow channels (10–12). Athabasca Valles itself extends ~300 km southwest from the Cerberus Fossae, where it widens into Cerberus Palus (Fig. 1). The large

fractured plates in Cerberus Palus—hereafter referred to as primary plates—have a rubbly texture, low thermal inertia, and appear to be

mantled by a sufficient quantity of aeolian sand and dust to obscure the underlying material from spectral analysis (13, 14). The relative motion between primary plates may be implied from their geometry (i.e., fitting the plates together like a jigsaw puzzle). The interplate terrain is patterned with meter-scale polygons with raised centers, has higher thermal inertia than the primary plates, and appears to be mantled by a thinner assemblage of aeolian fines. Such polygonal ground patterns can have either a periglacial or volcanic origin (2–4, 15, 16). Unique surface morphologies detected in imagery acquired by the High Resolution Imaging Science Experiment (HiRISE) on board the Mars Reconnaissance Orbiter (MRO), however, provide strong evidence for a volcanic origin.

Patterned terrain may form in periglacial environments on Earth and Mars (2–4), where tensile stresses caused by seasonal fluctuations in ground temperature have formed polygons in

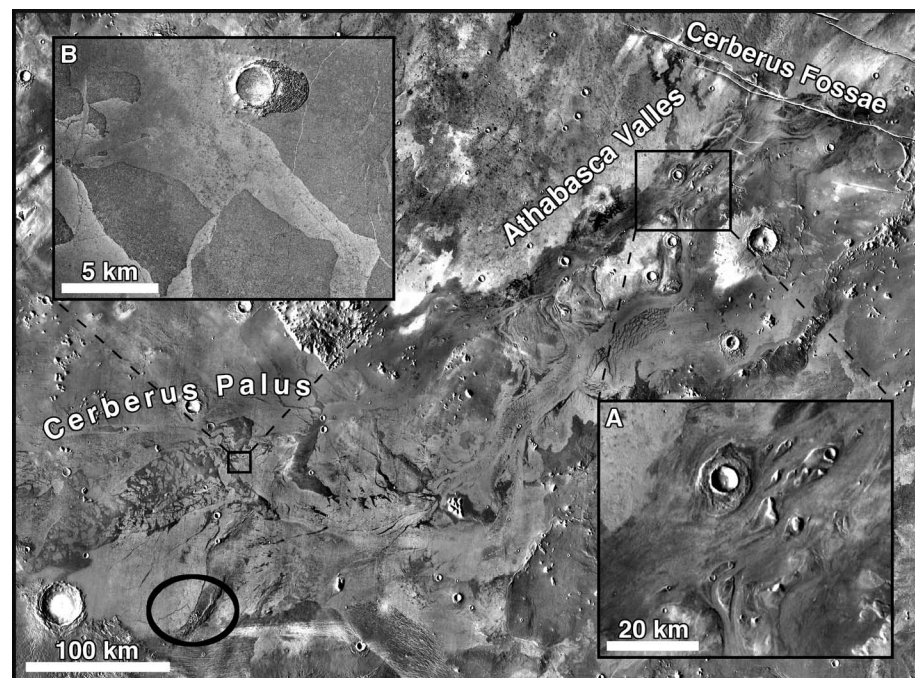


Fig. 1. Thermal Emission Imaging System (THEMIS) daytime infrared mosaic of the Athabasca Valles region. Dark oval shows location of study area detailed in Fig. 3. (A) Streamlined landforms in upper Athabasca Valles. (B) Details of Cerberus Palus primary-plate motion in Context Camera (CTX) image P02_001791_1852.

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ice-rich regolith (17). The Phoenix Lander first confirmed the presence of martian ground ice at 68° N in polygonal terrain (18). Patterned ground has since been mapped in all regions above 30° N latitude and below 30° S latitude (19). Cerberus Palus, however, is located at ~5° N and is not included in such surveys. The stability of shallow subsurface ice at these low latitudes remains controversial (20, 21).

Volcanic processes may also produce polygonal features (15, 16, 22). For example, freshly exposed molten lava on the surface of lava lakes and stagnated lava flows in Hawaii may cool within minutes to form a thin plastic crust, which rapidly cracks into large polygonal plates. As these plates cool and contract, they continue to subdivide into smaller polygons. During this process, gasses from the underlying molten lava rise to accumulate under the crustal polygons and preferentially escape through the cracks. This peripheral gas escape allows the crust around the cracks to sag while the trapped gas maintains the polygons' positive central buoyancy, leading to the final high-centered morphology. On Earth, the final generation of polygons are typically ~5 m wide, with as much as 0.5 m of relief (15, 16).

Polygon forms are also observed within inflated terrestrial lava flows. Thin, cool crusts of lava serve as insulators for underlying hot liquid flows. This thickening crust eventually becomes strong enough to retain incoming lavas, increasing the hydrostatic head and resulting in uniform uplift of the entire sheet flow (23, 24). Large (~1 to 3 m), up-bowed pahoehoe plates that are bounded by cooling fractures, observed in Hawaii, may have formed on ponded flows that were later inflated by such processes (22).

HiRISE image PSP_007250_1840 contains ~4 km² of well-preserved, relatively dust-free interplate patterned ground that is typical of the Athabasca region (Fig. 2). The interplate terrain here is arranged in a network of "troughs" bordered by primary plates and a raised ejecta blanket from an earlier impact. High-centered polygons here are 5 to 15 m in diameter. Within these troughs, we have identified numerous spiral patterns, ranging in diameter from 5 to 30 m (Fig. 2). They are typically doubly voluted (like the letter "S") but are singly voluted in some instances. These spirals are crosscut by the polygon-forming cracks in nearly all cases (e.g., Figure 2C). In total, 269 spirals have been identified in this image (Fig. 3). Of these, 174 spirals have a clockwise-in orientation, 43 are counterclockwise-in, and 52 circular features remain unclassified due to limits in resolution. Many additional HiRISE images were examined, but only two others were found to contain a small number of spirals. The HiRISE camera will likely detect additional spirals as image coverage in this region increases over time.

The spiral patterns in Cerberus Palus are morphologically consistent with lava coils on Earth that form on the surface of active and stagnated pahoehoe lava flows and lava lakes

(25, 26). Terrestrial lava coils range from 5 cm to at least 10 m in diameter. Two types of coils have been observed in terrestrial lava flows, each with slightly different characteristics and formation processes. The first forms along distinct shear zones between two blocks of crust above flowing lava. Strips of crust are pulled away and physically coiled from the differential motion. These coils are typically small (1 m or less in width) and may have centers higher, lower, or coplanar with the margin. The second,

larger form also develops in shear zones where wrinkles in thin congealed lava skins or molten lava exuded through ephemeral shear cracks rotate slowly. The rotation is likely caused by a Kelvin-Helmholtz instability in the underlying flow, where two fluids of different velocity or density flow past each other (27). With few exceptions, terrestrial lava coil orientation is consistent with the direction of shearing, although such shears are commonly not preserved: Clockwise-in coils record right-lateral shear,

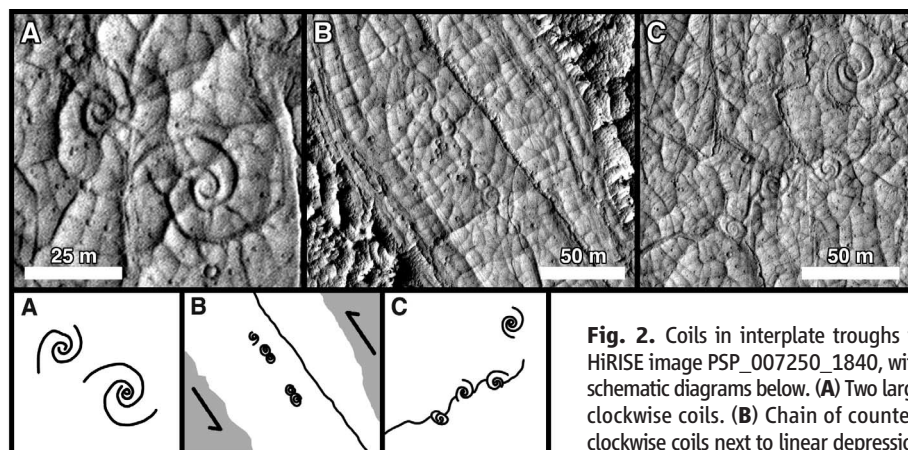
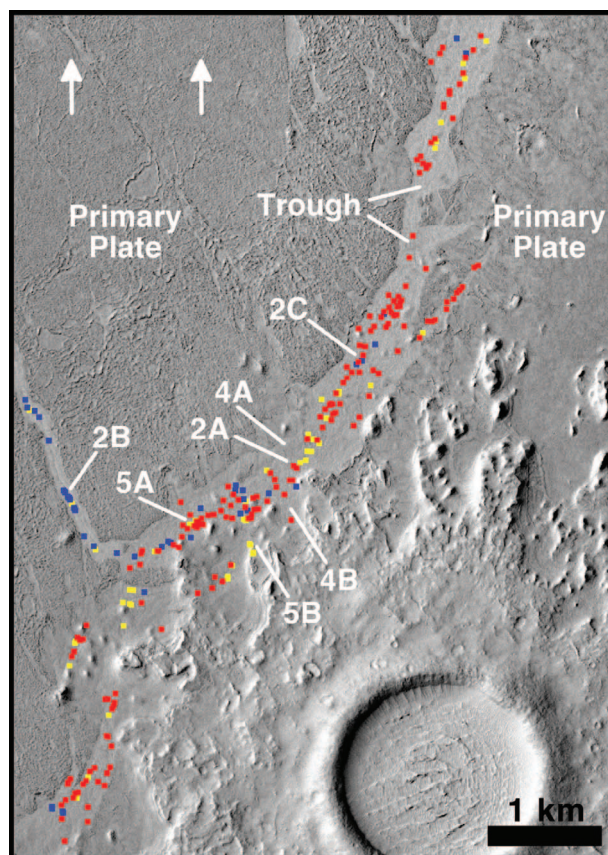


Fig. 2. Coils in interplate troughs in HiRISE image PSP_007250_1840, with schematic diagrams below. (A) Two large clockwise coils. (B) Chain of counter-clockwise coils next to linear depression in the northwest-southeast trending

trough. Primary plates are visible in periphery, with direction of shear indicated by arrows in diagram. (C) Large clockwise coil in upper right is crosscut by polygon fractures. Smaller coils with mixed orientations along bottom-center are connected by meandering depression.

Fig. 3. CTX image P16_007250_1841 (~4.1° N, 150.1° E) indicates the location of coils in HiRISE image PSP_007250_1840. Primary plates appear dark-toned and rough, whereas troughs are light-toned and smooth. Colored markers show coil location and orientation: red, clockwise-in; blue, counterclockwise-in; yellow, undetermined. Location of Figs. 2, 4, and 5 are indicated with lines. Arrows show general direction of plate motion inferred from plate geometry and coil orientation.



whereas counterclockwise-in coils record left-lateral shear (25, 26).

Both types of subaerial lava coils are present in HiRISE image PSP_007250_1840. They are concentrated near the centers of ~50- to 800-m-wide troughs. Chains of small, like-oriented coils (~4 m wide) with raised centers appear to be present along linear shear zones (Fig. 2B). Such coils may be voluted strips of plastic crust, as described above. Within the smaller northwest-southeast trending trough, coil chains are counterclockwise-oriented (e.g., Figure 2B), indicative of a left-lateral shearing pattern. The larger (>10 m wide), coplanar lava coils are more likely formed by means of the latter mechanism discussed above. These coils are abundant within the wide, southwest-northeast trending trough. Here, the orientations are mixed but predominantly clockwise, indicative of more

complex flow patterns with a less substantial overall right-lateral shear.

The troughs themselves appear to have fractured into “secondary plates” that drifted apart in several locations (Fig. 4). As with the primary plates, the direction of secondary-plate movement may be inferred from the plate geometry. When the pieces of the puzzle are reassembled, it is also apparent that the polygons and coils on these plates are continuous across the fracture zones and that these surface features must have been present before this secondary-plate drift occurred. The overall patterns of the primary and secondary plates’ motion are consistent with directions of shear inferred from spiral orientation.

All of the aforementioned features and inferred processes are best explained by volcanic, rather than ice, processes. The primary plates

likely formed as an early solidification crust atop a lava flow. As this crust fractured and drifted apart, underlying molten lava was exposed. Lava coils formed on the cooling surface in the shear zones between the primary plates. Some coils were eventually preserved in the flow crust as it fully solidified and fractured into the network of polygons that we observe in the troughs today. In several locations, these secondary, patterned plates then fractured into smaller plates, thus exposing more molten lava and allowing the process of lava coil and crust formation to repeat.

Ice-related processes fail to account for our observations. There are no known mechanisms to naturally produce spiral patterns in ice-rich environments on the scale and frequency observed in our study area. If the primary plates were ice rafts, then the water extruded during rafting would not have the correct viscosity to form and preserve the coils. We have also shown that the polygons formed after the fracture and drift of the secondary plates on which they are present. This precludes the notion that the polygons formed over decades or even centuries in an ice-rich regolith, because it seems unlikely that frozen soil could somehow fracture and drift.

The patterns of the primary and secondary plates’ movement are morphologically similar to historical platy-ridged Icelandic lava flows (28). The troughs also contain other features that are indicative of volcanism, such as sinuous pressure ridges (Fig. 2C). In several locations, the secondary plates have been pushed up from below, sometimes causing them to crack (Fig. 5). We suggest that these are tumuli or evidence of flow deflation and draping of the plates on underlying topographic highs (23). A large fracture on one such feature exposes the edge of a secondary plate that is ~2.5 m thick (Fig. 5B).

The secondary plates contain many linear to meandering depressions up to 10 m wide (Fig. 2B). One depression runs across the entire observable length (~2 km) of the narrow trough in Fig. 2A and is interpreted to be an incipient rift in a secondary plate. The chain of coils in Fig. 2C are connected by curvilinear depression in an arrangement similar to wax models of rotating microplate formation in sea-floor spreading centers (29, 30). This spreading-center style of plate formation, on a smaller scale, may have led to coil and secondary-plate formation in areas like Figs. 2C and 4, where minor lateral translation influenced coil orientation.

We conclude that the lava coils, polygons, and primary/secondary plates in Cerberus Palus are primary volcanic structures. It is likely that lava coils and evidence of secondary-plate motion are fairly common in Cerberus Palus. Despite differences in aeolian cover, the primary and secondary plates are presumed to have developed within contemporaneous flow events. However, it is unclear whether the multiple generations of

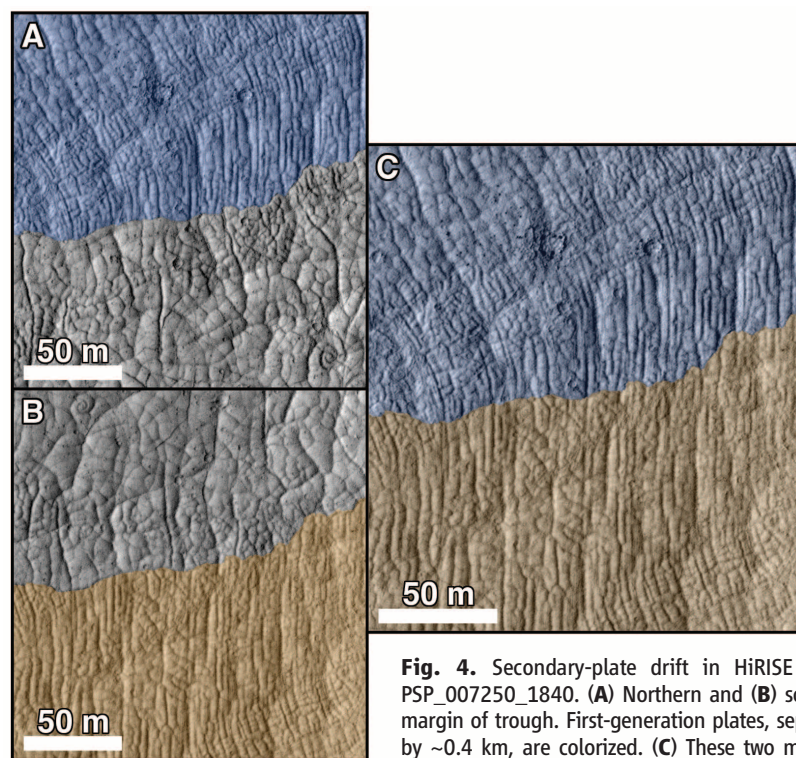


Fig. 4. Secondary-plate drift in HiRISE image PSP_007250_1840. (A) Northern and (B) southern margin of trough. First-generation plates, separated by ~0.4 km, are colorized. (C) These two marginal plates are continuous when sutured together.

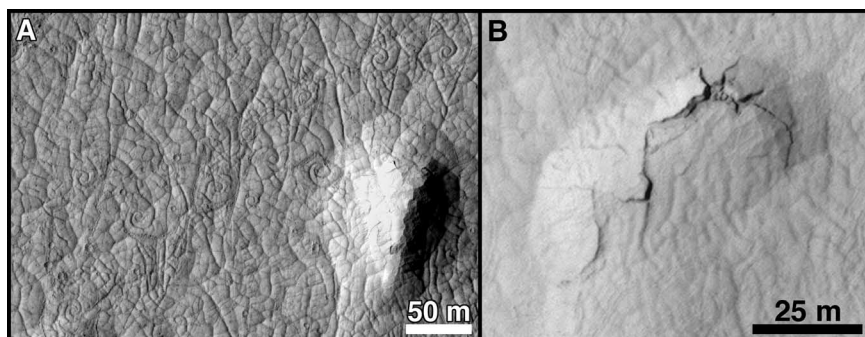


Fig. 5. HiRISE image PSP_007250_1840. (A) Raised crust next to complex chain of clockwise and counterclockwise coils. (B) Edge of uplifted crust is fully exposed.

plates are evidence of multiple distinct surges of lava or one continuous flow with some temporal variation in flux or flow path. Nonetheless, the features here are very well preserved, indicating that Cerberus Palus is either very young or was buried shortly after formation and recently exhumed.

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Organic Synthesis via Irradiation and Warming of Ice Grains in the Solar Nebula

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Complex organic compounds, including many important to life on Earth, are commonly found in meteoritic and cometary samples, though their origins remain a mystery. We examined whether such molecules could be produced within the solar nebula by tracking the dynamical evolution of ice grains in the nebula and recording the environments to which they were exposed. We found that icy grains originating in the outer disk, where temperatures were less than 30 kelvin, experienced ultraviolet irradiation exposures and thermal warming similar to that which has been shown to produce complex organics in laboratory experiments. These results imply that organic compounds are natural by-products of protoplanetary disk evolution and should be important ingredients in the formation of all planetary systems, including our own.

Complex organic compounds have been found in meteorites and interplanetary dust particles, leading many to suggest that Earth's prebiotic material was delivered by comets and chondritic planetesimals. The origin of these organic compounds, however, is unknown. Whereas organic molecules can be produced during aqueous alteration of a parent body (1) or through Fischer-Tropsch reactions in protoplanetary disks (2), the high D/H ratios found in meteoritic organics point to a low-temperature origin (3). Irradiation of astrophysical ice analogs (containing simple molecules like H₂O, CO, CO₂, NH₃, and CH₃OH) by ultraviolet (UV) photons in the laboratory has produced a suite of organic molecules similar to those found in primitive bodies in our solar system, including amino acids, amphiphiles,

quinones, and nucleobases (4–10). These experiments simulated conditions (temperatures, pressures, radiation fluxes) similar to those found in dark molecular clouds—the collection of gas and dust that collapses to form stars and planets. Within these clouds, ice-mantled grains are exposed to relatively low fluxes of UV from the ambient interstellar radiation field, allowing organics to be produced. It remains unclear, however, if the full suite of complex organics found in primitive bodies could be produced within a molecular cloud and to what extent the compounds were preserved during the formation of the solar system.

These experimental conditions are also relevant to the outer solar nebula, where temperatures were low enough for C-, H-, O-, and N-bearing ices to form (11). If ices in this region were irradiated to substantial levels, organics would have been produced, increasing the inventory of such compounds beyond what may have been inherited from the molecular cloud. Indeed, observations of molecular emission features suggest that protoplanetary disks support

active organic chemistry, processing the raw materials from the molecular cloud to yield higher abundances of organic species, though the pathways for production are unclear (12).

To investigate the production of organics after molecular-cloud collapse, we modeled the paths of ice-mantled grains through the solar nebula (Fig. 1) (13–16). Particles migrated around the nebula due to disk evolution, gas drag, gravitational settling, and both radial and vertical turbulent diffusion. We assumed that the level of turbulence in the disk is characterized by the turbulence parameter, α , which describes both the viscosity in the disk, $\nu = \alpha c_s H$ where c_s is the local speed of sound in the disk and H is the local gas scale height, and the diffusivity of trace materials, $D \sim \nu$ (16, 17). We set $\alpha = 0.001$ everywhere, as the magnetorotational instability is expected to have operated in the outer disk (18).

The disk was assumed to be in a steady state and had a surface density distribution

$$\Sigma(r) = 2000 \left(\frac{r}{1 \text{ AU}} \right)^{-1} \text{ g/cm}^2 \text{ [amounting to}$$

$1/_{10}$ of the solar mass out to 70 astronomical units (AU)] and a thermal structure $T(r) = 200 \left(\frac{r}{1 \text{ AU}} \right)^{-1/2}$ K (where r is the distance from the Sun), appropriate for flared disks around young T Tauri stars (11, 19). We tracked the dynamics of 5000 ice-mantled grains with radius $a = 1 \mu\text{m}$ and unit density. Although particles of a broad range of sizes would have been present, we chose to follow 1- μm particles as individual organic hot spots, and globules in meteorites and comets are often found to be of this spatial scale (20, 21). Particles began at the midplane of the disk (with the height above the disk midplane given by z , and the midplane defined as $z = 0$ AU) where $T = 30$ K ($r = 49$ AU). At this temperature, C- and N-bearing ices of the type needed to form organics condensed (22), and D/H ratios in water are elevated due to ion-molecule reactions (23).

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Fig. 1. (Left) Trajectory of one of the surviving particles (which are defined as those that did not migrate inside of 0.1 AU of the young Sun) through the solar nebula superimposed on a map of the UV radiation flux in the disk. The flux is normalized to its value at $z = \pm\infty$ and assumed to propagate normal to the disk midplane. The blue and green symbols denote the starting and final locations, respectively. **(Right)** The r and z coordinates of the particle through time.

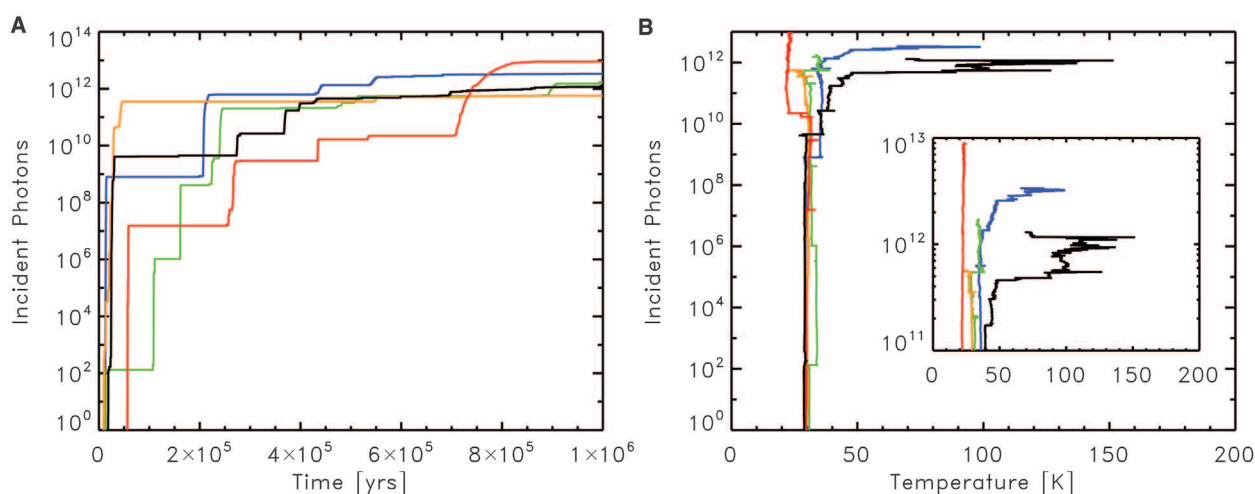
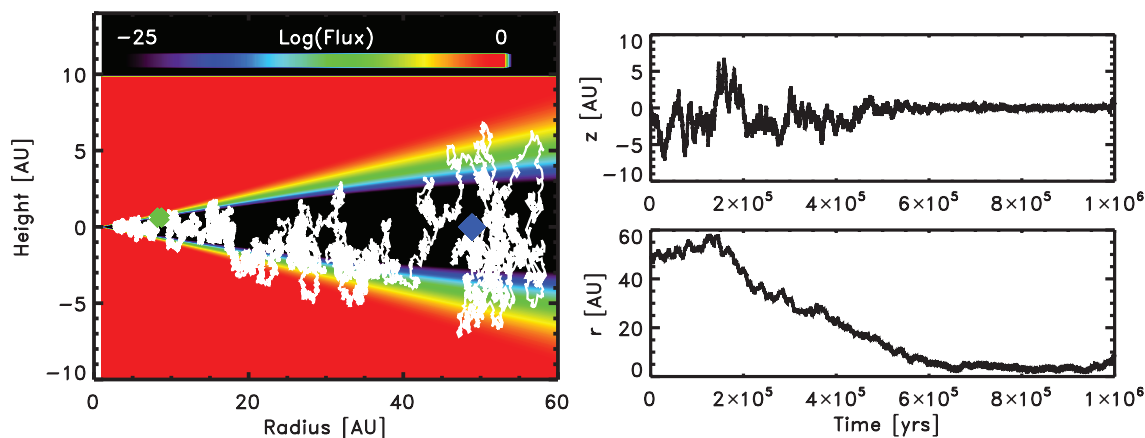


Fig. 2. (A) Cumulative number of photons incident on five grains throughout the 10^6 -year lifetime in the disk, assuming an incident flux of $1G_0$. The black curve corresponds to the grain whose trajectory is shown in Fig. 1; the other

curves represent other random particles from the simulation. **(B)** Total incident photons seen by the same particles plotted against their temperatures. The inset shows a zoomed-in view.

As a particle moved through the disk, we calculated the number of UV photons incident on its surface. The radiation was assumed to be incident on the disk with a flux, F_0 , directed normal to the disk midplane. The flux was attenuated with depth as photons were absorbed by solid particles. Thus, the flux everywhere was $F(r, z) = F_0 e^{-\tau(r, z)}$, where τ is the optical depth of material suspended in the disk above the point of interest [$\tau(r, z) = \int_{|z|}^{\infty} \rho_g(r, z) \kappa dz$, where $\rho_g(r, z)$ is the density of material in the disk and κ is the average opacity of the suspension]. We assume an opacity of $\kappa = 7.5 \text{ cm}^2/\text{g}$, a conservative value that is on the high end of the range expected in the cool outer regions of a protoplanetary disk (24). Lower opacities would increase the number of photons seen by each particle. We take as a baseline an incident flux equivalent to the current interstellar UV flux of $10^8 \text{ photons cm}^{-2} \text{ s}^{-1}$ ($1G_0$) (25), but higher fluxes can be considered by multiplying by the desired enhancement factor.

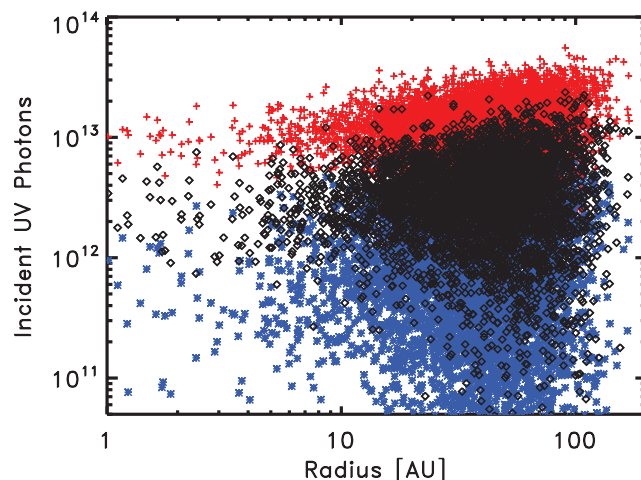
The starting location of the particles was shielded such that the flux was attenuated by

more than 25 orders of magnitude. As most of the mass of the nebula was located around the midplane, these conditions are often used in calculating the chemical evolution of primitive materials. At this location, an ice grain would have seen $< 10^{-25} F_0 \pi a^2 \Delta t \sim 10^{-11}$ photons if it remained at its starting location throughout the simulation, a period of $\Delta t = 10^6$ years. However, in the presence of vertical diffusion, small particles spend $\sim 68\%$ of their time within $1H$ of the midplane but 32% of their lifetimes at higher altitudes, with $\sim 5\%$ being spent above $2H$ (14). Because the UV flux was a strong function of height, it was the infrequent but non-negligible excursions to high altitudes that were primarily responsible for small grains seeing large numbers of photons. Irradiation exposures also increased as grains diffused outward in the disk, where the lower surface densities of gas and dust allowed the UV to penetrate closer to the disk midplane. Thus, the radial and vertical motions of the grains controlled their UV exposures.

Figure 2 shows that the cumulative number of UV photons grains encountered did not climb uniformly. Rather, the increases in incident photons were episodic and occurred when a grain was lofted to high altitudes or far out in the disk. Plotting the irradiation histories against the temperatures seen by the particles, where we took the particle temperature as being equal to the gas temperature everywhere, demonstrates how the UV exposure generally rose early while the grains were cold and when all key ices remained on the grain. Less than 0.5% of photons would desorb these molecules (26), which would then recondense on the same or another grain. Thus, loss of mass in this manner would be minimal.

Figure 3 shows that a typical dosage for the particles was $\sim 5 \times 10^{12}$ photons in our nominal case. Assuming a pure H_2O grain, this corresponds to ~ 50 UV photons per molecule. Experimental results suggest an efficiency of production of approximately one organic molecule produced per 400 incident photons (6, 16). Thus $>12\%$ of the mass of ices in the outer solar nebula would

Fig. 3. Total number of incident photons for each of the 4979 surviving grains plotted against their locations after 10^6 years. A typical exposure for our nominal flux (black diamonds) is $\sim 5 \times 10^{12}$ photons. The red and blue symbols represent the same calculations using the lower and higher opacities, respectively, as described in the text. The variations in average flux due to opacity differences are less than one order of magnitude.



be converted to organic compounds. However, protoplanetary disks like our solar nebula are exposed to enhanced UV fluxes, either from their own central stars (27) or from other stars in a low-mass cluster, resulting in average incident fluxes of up to $\sim 10^3 G_0$ (28). This increases the average dosage of ice grains to 50,000 photons per molecule, greatly increasing organic production.

Grain growth would have resulted in lower photon dosages due to decreased particle cross sections per unit volume (16, 29). However, the decrease in opacity of the nebula would have allowed UV photons to penetrate deeper into the disk (29) to irradiate the grain aggregates. Further, models of dust growth show that fragmentation would constantly free particles from the larger aggregates through energetic collisions (30). Thus, even if icy grains remain as 1- μm entities for just a part of the 10^6 years modeled here, they can receive substantial UV dosages (Fig. 2).

By itself, irradiation does not produce the organic compounds. When UV photons are incident on astrophysical ices in the laboratory, they break molecular bonds in the ices, producing reactive ions and radicals. If these reactive photoproducts are adjacent to each other, they may react to form new compounds, even at very low temperatures. When the irradiated ice is warmed, the photoproducts become mobile and react more readily. Such reactions occur during warm-ups through any temperature range but are particularly enhanced during the amorphous-amorphous transition near 80 K, the amorphous-to-cubic transformation around 135 K, and the sublimation of the H_2O in the ice at $T > 135$ K (4–10, 16). Thus, warming of irradiated ices yields a burst of organic synthesis, even if the ice is not currently being irradiated. Reactions brought on by warming are still largely constrained by proximity rather than considerations of normal chemical equilibrium. However, these experimental results are robust in that the amounts and types of molecules produced are largely insensitive to the temperature of the ices during ir-

radiation and the exact composition of the starting ices (16).

Particle warming would have occurred as grains diffused to high altitudes where the increased photon flux would have heated grains beyond the midplane temperature. Though we assumed our disk was isothermal with height, temperatures may reach ~ 10 to 50 K above midplane temperatures at heights of $|z| > 0.2r$ outside ~ 10 AU (31). As such, not only would particles accumulate substantial UV dosages, but they would also be warmed as they moved through the disk. Thus, the conditions shown to produce organics in laboratory experiments, irradiation and warming, would be natural consequences of the dynamical evolution of ices in the solar nebula. Photo-processing in the solar nebula thus represents an ideal environment for this form of organic synthesis.

The grains in our study are irradiated during their large-scale movement in the disk, which suggests that similar products would be made available to meteoritic and cometary bodies, consistent with observations (20, 21). Further, organics on particles brought to the innermost regions of a protoplanetary disk would vaporize, allowing the products of this organic chemistry to be observed in the gas phase, offering an explanation for the emission features observed by the Spitzer Space Telescope (12). As we predict that complex species such as amino acids will be formed, identification of more complex organic species in other disks may serve to test this method of production. Because the total UV fluences received by ices in the solar nebula are considerably greater than those experienced by typical ices in dense molecular clouds, disk processing would yield greater overall conversions of ices into organics and would produce organics of greater complexity. The exact mass of organic production remains uncertain, as it will depend on the incident UV flux and the mass of ice contained in the outer disk. Our results, however, demonstrate that cold, icy 1- μm grains would see UV dosages of $\sim 10^{12}$ to 10^{15}

photons for modest incident fluxes of 1 to $10^2 G_0$. This allows for substantial organic production, with at least $\sim 5\%$ of the mass of ices being converted to organics and production being limited more by the availability of starting materials than by energetic photons (16). These fluxes are much less than those thought to be necessary for organic production to occur in protoplanetary disks ($\sim 10^6 G_0$) (29). Thus, rather than being limited to disks around stars located near massive OB stars, complex organics should be produced in disks around all stars and made available for delivery to the surface of planets where they could play a role in the origin of life.

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Supplementary Materials

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Ocean Salinities Reveal Strong Global Water Cycle Intensification During 1950 to 2000

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Fundamental thermodynamics and climate models suggest that dry regions will become drier and wet regions will become wetter in response to warming. Efforts to detect this long-term response in sparse surface observations of rainfall and evaporation remain ambiguous. We show that ocean salinity patterns express an identifiable fingerprint of an intensifying water cycle. Our 50-year observed global surface salinity changes, combined with changes from global climate models, present robust evidence of an intensified global water cycle at a rate of $8 \pm 5\%$ per degree of surface warming. This rate is double the response projected by current-generation climate models and suggests that a substantial (16 to 24%) intensification of the global water cycle will occur in a future 2° to 3° warmer world.

A warming of the global surface and lower atmosphere is expected to strengthen the water cycle (1–3), largely driven by the ability of warmer air to hold and to redistribute more moisture. This intensification is expressed as an enhancement in the patterns of surface water fluxes [evaporation and precipitation (E-P)] and, as a consequence, ocean surface salinity patterns. According to the Clausius-Clapeyron (CC) relation and assuming a fixed relative humidity, we expect a $\sim 7\%$ increase in atmospheric moisture content for every degree of warming of Earth's lower troposphere (2). Of greatest importance to society, and the focus of this work, is the strength of the regional pattern of E-P, which in climate models scales approximately with CC, whereas global precipitation changes more slowly at a rate of 2 to $3\% \text{ } ^\circ\text{C}^{-1}$, limited by tropospheric energy constraints (2, 4).

An intensification of existing patterns of global mean surface E-P is found along with enhancements to extreme events such as droughts and floods (1, 5) in available 21st-century climate projections, forced by anthropogenic greenhouse gases (GHGs) from the Coupled Model Intercomparison Project Phase 3 [CMIP3 (6)]. This has been labeled the “rich get richer” mechanism, where wet areas (compared with the global mean) get wetter and dry regions drier (7). There is, however, little consistency

in the seasonal changes provided by model projections and poor agreement when compared with regional observational estimates (8). Additionally, atmospheric aerosols included in these projections can regionally counteract the GHG-driven warming and act to suppress the local water cycle through dynamical changes (9, 10).

Given the above broad-scale model responses and the CC relationship, an intensification of $\sim 4\%$ in the global water cycle (E-P) is expected to already have occurred in response to the observed 0.5°C warming of Earth's surface over the past 50 years (11). However, obtaining a global view of historical long-term rainfall pattern changes is made difficult because of the spatially sparse and short observational record. Long, high-quality land-based records are few and Northern Hemisphere-biased (12). Direct high-quality long-term rainfall estimates over oceans [which comprise 71% of the global surface area and receive over 80% of global rainfall (13) (fig. S1)] are very scarce, with most global observational products dependent on data contributions from satellites, themselves sensitive to error (14, 15). Additionally, because of the short temporal coverage (~ 15 to 30 years) by satellite missions, trends are likely affected by natural decadal modes of variability and may dominate much of the measured changes (3). This challenge is exacerbated by the spatially and temporally sporadic nature of rainfall, making the derivation of broad-scale averages of small multidecadal changes from a sparse network of observing stations error-prone (16). These difficulties are evident in the differing signs of long-term trends between reconstructed rainfall data sets (17, 18). Discrepancies among air-sea evaporative flux products (19) undermine their use in resolving long-term water cycle changes. As a result, we do not yet have a definitive view on whether Earth's water cycle has intensified over the past

several decades from atmospheric observing networks (12, 20).

It has long been noted that the climatological mean sea surface salinity (SSS) spatial pattern is highly correlated with the long-term mean E-P spatial pattern (21) (Fig. 1, A and D), reflecting the balance between ocean advection and mixing processes and E-P forcing at the ocean surface (21–23). Several studies of multidecadal SSS changes reveal a clear pattern where increasing salinities are found in the evaporation-dominated midlatitudes and decreasing salinities in the rainfall-dominated regions such as the tropical atmospheric convergence zones and polar regions (22, 24–28). These previous studies have used optimally averaged pentadal historical ocean data (24) or the difference between pre-2000 and post-2000 climatologies (27), the latter period being strongly supported by the modern baseline provided by the Argo Programme (29) to investigate long-term salinity changes in the global ocean. By using a direct local fit of trends to historical and Argo data simultaneously (25), we map the multidecadal linear SSS trends back to 1950 (Fig. 1, D and G). Over the last 50 years, SSS changes reflect an intensification of the mean SSS patterns. This strong and coherent relationship is expressed through the high spatial pattern correlation coefficient (PC) of ~ 0.7 (fig. S2) between the mean SSS and independent estimates of long-term SSS change. Following the “rich get richer” mechanism (7), salty ocean regions (compared to the global mean) are getting saltier, whereas fresh regions are getting fresher (24–28). This robust intensification of the observed SSS pattern is qualitatively consistent with increased E-P if ocean mixing and circulation are largely unchanged.

In trying to quantitatively relate SSS changes and E-P changes, previous studies have made strong simplifying assumptions. One estimate of a global 3.7% E-P intensification from the 1970s to 2005 (27) is based on the assumption of an unchanging ocean mixing and advection field, with the additional assumption that no salt or freshwater exchange has occurred over this time with the ocean below 100 m. However, several studies have shown that subsurface salinity changes have occurred during the 20th century (24, 25), with many of the largest signals expressed at depths greater than 100 m. Another study used subsurface salinity changes on isopycnals to deduce E-P changes at the surface density outcrops (26). This approach is error-prone because broad-scale salinity changes on density surfaces can largely be explained by the subduction of broad-scale warming and not E-P changes alone (25). To avoid such strong assumptions and explore the use of SSS pattern changes as a water cycle diagnostic, we used the most comprehensive simulations available to date of the historical and future global climate: the CMIP3 simulations of the 20th century (20C3M) and the Special Report on Emissions

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Scenarios (SRES) 21st-century future projections (6). Within these simulations, we investigated the relationship between SSS and E-P pattern changes. These simulations capture the full range of complex dynamical changes in response to GHG forcing, which include ocean surface (and subsurface) temperature changes, dynamical shifts to ocean and atmospheric circulation, upper ocean stratification changes, as well as the regional effects of aerosols on water cycle operation.

To quantify and compare the strength of broad-scale SSS pattern intensification in both observations and CMIP3, we formed zonal ocean basin (Pacific, Atlantic, and Indian) averages for both the 50-year (1950–2000) climatological mean SSS and its 50-year (1950–2000) linear trends. A linear regression was undertaken by using the basin zonal averages of the climatological mean SSS (x axis) anomaly from the global climatological SSS against the SSS change pattern (y axis; Fig. 1J). We defined the resulting slope of this relationship as the pattern amplification (PA) and the corresponding correlation coefficient (R) as the PC. A key advantage of this analysis is its insensitivity to the mean spatial climatological biases in model fields (30) when compared to observations, because the model change fields are compared to their own model climatology. We formed the PA and PC metrics for each model simulation

and our observational analysis (fig. S2 presents comparative basin zonal mean analyses for available global SSS studies; see supplementary text section 1.1).

Analysis of trends in observed SSS indicates that from 1950 to 2000 the SSS PA is 8% with a PC of 0.7 (Fig. 1J). Similar to observations, many models show a high PC (~ 0.7 to 0.9) between the climatological mean SSS and the corresponding climatological mean E-P. However, most 20C3M model simulations show a weaker-than-observed spatial PC and PA between the 50-year SSS mean and SSS change patterns (Fig. 1, D and G versus E and H and F and I) and do not uniformly provide a realistic simulation of observed surface mean SSS patterns or their change over 1950–2000 (Fig. 1, D and G; fig. S6; table S2; and supplementary text section 2). Our examples of the simulations that most closely replicate the observed spatial change and mean patterns (Fig. 1, E and H) and those that produce an almost inverse spatial change pattern (Fig. 1, F and I) compared with the observed results (Fig. 1, D and G) illustrate the range of responses found in CMIP3 (Fig. 1). Some models show similar numbers to those observed (Fig. 1, E, H, and K), whereas others have very low values of both PA and PC (Fig. 1, F, I, and L), indicating no clear SSS pattern amplification. Such discrepancies raise a key question: What

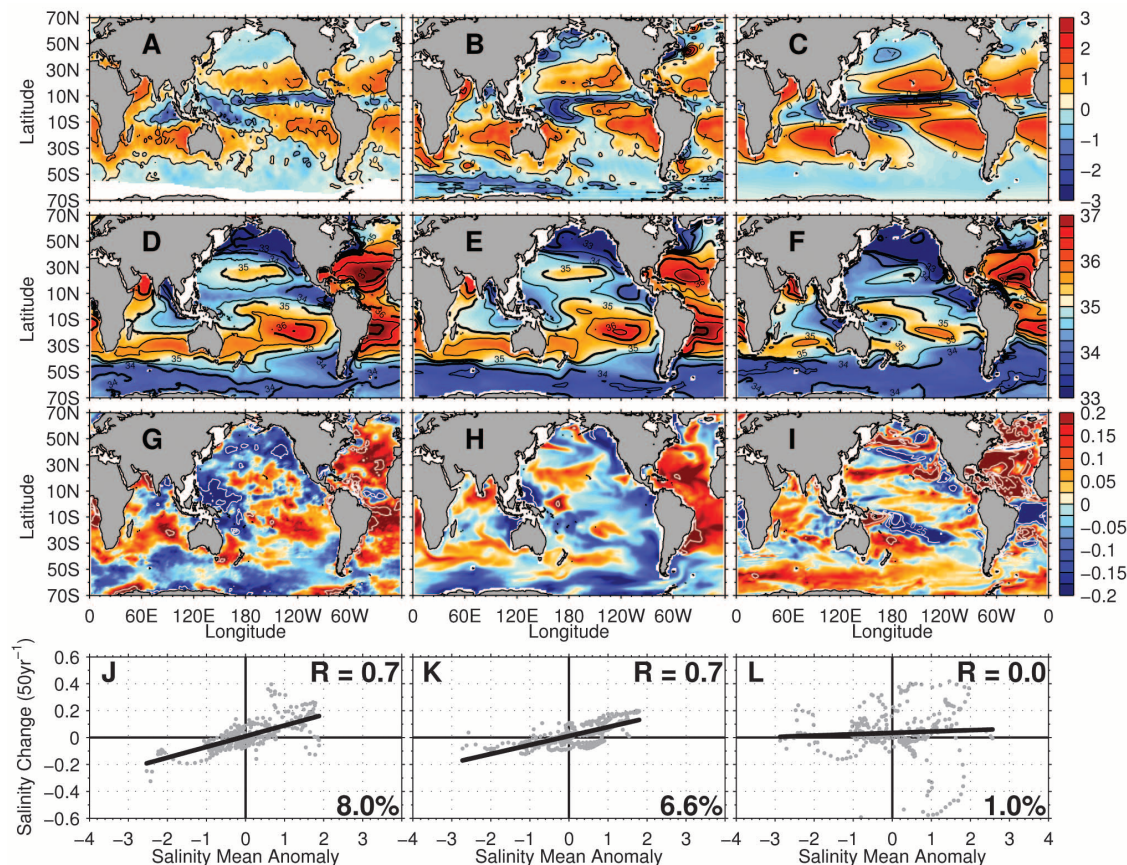
controls this difference in the modeled SSS response, and how is this related to water cycle changes?

The PA and PC methodology can also be used when considering other variables, such as the surface water flux (E-P). CMIP3 simulations show a relationship between SSS PA and the E-P PA (Fig. 2B). This key result supports the use of SSS PA as a diagnostic of a changing water cycle and also provides a relationship in which to consider the observed SSS PA for 1950–2000. The CMIP3 SSS patterns amplify at twice the rate of E-P patterns (Fig. 2B). The reason that E-P PA drives a stronger response in SSS PA for CMIP3 is not clearly understood and requires further investigation, but the relationship between them is compelling.

When investigating water cycle changes, it is important to consider the coincident global surface warming, the natural thermodynamic framework of water cycle amplification. If expressed in a per degree warming context, such water cycle rate changes can then be directly compared to other studies, both oceanographic and atmospheric in their origins (table S3, fig. S9, and supplementary text section 4).

For both the 20C3M and SRES CMIP3 simulations, we find a relationship between the rate of global average surface warming (ΔT_a) and the rate of SSS PA and PC strength (Fig.

Fig. 1. Observed and selected CMIP3 20C3M simulations of surface salinity and water fluxes. Surface annual mean water flux from 1950 to 2000 (E-P m year^{-1}) (A to C). Surface annual mean salinity 1950–2000 (PSS-78) (D to F). Surface salinity change 1950–2000 (PSS-78 50 year^{-1}) (G to I). Basin zonal-mean surface salinity trends (y axis) versus basin surface mean salinity anomaly from the surface basin zonal mean; in text these are the PA (slope) and PC (correlation coefficient, R) (J to L). The observed result of (25) [(D), (G), and (J)] and (A) the observed result of (32) for 1980–1993. Results from the Canadian Centre for Climate Modelling and Analysis, CGCM3.1 (T63) model [(B), (E), (H), and (K)] and results from the UK MetOffice, HadGEM1 model [(C), (F), (I), and (L)]. For (A) to (C), black contours mark every 1 m year^{-1} . For (D) to (F), black contours represent surface mean salinity every 1 PSS-78 for bold lines and 0.5 for thin. For (G) to (I), white contours represent surface salinity change every 0.25 PSS-78.



2A). The 20C3M simulations in which the warming rate is low (generally those with comprehensive aerosol schemes; contrast diamonds and circles in fig. S5, table S1, and supplementary text section 2.2) feature low SSS PA, with spatial change patterns having only slight correspondence to the spatial mean pattern and consequently a low SSS PC (illustrated by the simulation in Fig. 1, F, I, and L). The stronger warming SRES simulations express a clearer and larger pattern amplification response ($PC > 0.5$) than most 20C3M simulations (Fig. 2A). The increase in PC with enhanced PA suggests a signal-to-noise process is operating, whereas in weakly warming simulations model internal variability dominates the change signal. A PC-weighted line of best fit through the 93 CMIP3 simulations suggests that SSS patterns intensify with warming at $8\% \text{ } ^\circ\text{C}^{-1}$ (Fig. 2A), which is half of our 1950–2000 observed rate ($16\% \text{ } ^\circ\text{C}^{-1}$; Fig. 2A). As expected on the basis

of past analyses of CMIP3 (2), the E-P PA is also linearly related to surface warming rates (Fig. 2C), with the model line of best fit below CC ($4.5\% \text{ } ^\circ\text{C}^{-1}$). Also in agreement with many previous analyses (1, 2), total global average rainfall is linearly related to warming rates but with a distinctively weaker slope than surface water flux, near $3.1\% \text{ } ^\circ\text{C}^{-1}$ (Fig. 2D). The stronger SSS PA response to warming and the tighter agreement among CMIP3 when compared with that for the E-P PA (Fig. 2, A versus C) suggest that long-term SSS pattern changes provide an identifiable, highly detectable, and particularly sensitive measure of long-term water cycle changes. It is likely that ocean mixing and circulation act to integrate and smooth the temporal and spatial patchiness of E-P fluxes at the ocean surface and provide a smoothed SSS anomaly field, which facilitates detection of broad-scale, persistent changes.

To independently demonstrate the strong relationship between 50-year salinity change and an enhanced water cycle, we explored the response of an ocean-only model to an idealized 5% E-P pattern increase. We used a version of the MOM3 ocean model, forced with E-P fields obtained from the National Centers for Environmental Prediction (NCEP) reanalysis. A linear trend in E-P was imposed to achieve a 5% increase over 50 years. The resulting spatial pattern of SSS change strongly mirrors the observed and CMIP3 ensemble mean patterns but with smaller absolute magnitudes (Fig. 3, A, C, and D versus B). The salinity pattern amplification is expressed for surface and subsurface changes (figs. S7 and S8 and supplementary text section 3). Therefore in a global ocean-only model, spatial salinity patterns enhance in response to an intensified E-P. A similar spatial response to the observed changes is found in CMIP3 but only for the strongly warming 20C3M simulations ($>0.5^\circ\text{C}$; Fig. 3, D versus C). Those simulations with less than the observed warming over 1950–2000 often incorporate aerosol effects that act to reduce warming (contrast diamonds and circles in fig. S5) and thus underpredict the subsequent water cycle amplification as expressed in SSS changes.

Despite their scatter, estimates from the CMIP3 ensemble show a weaker salinity pattern amplification per degree of warming ($8\% \text{ } ^\circ\text{C}^{-1}$; Fig. 2A) than has been observed ($16\% \text{ } ^\circ\text{C}^{-1}$; Fig. 2A). By using the modeled relationship between SSS PA and E-P PA from the CMIP3 ensemble [which shows that SSS PA increases at twice the rate of E-P PA (Fig. 2B)] and applying this relationship to our observed SSS PA estimate, we infer that over the past 50 years the global water cycle has amplified by 4%. Using the observed 0.5°C surface warming estimate (11), this inferred water flux amplification of $8\% \text{ } ^\circ\text{C}^{-1}$ is close to that predicted by the CC relationship ($\sim 7\% \text{ } ^\circ\text{C}^{-1}$). This rate of change is consistent with many other independent observational estimates (table S3 and fig. S9), which all provide evidence that an observed global water cycle amplification has occurred. However, CMIP3 ensemble averages of E-P PA produce a rate well below this of $4.5\% \text{ } ^\circ\text{C}^{-1}$ (Fig. 2C).

A change to freshwater availability in response to climate change poses a more important risk to human societies and ecosystems than warming alone. Changes to the global water cycle and the corresponding redistribution of rainfall will affect food availability, stability, access, and utilization. We show that ocean salinity is a particularly sensitive marker of water cycle change that provides us with a salty ocean–freshwater “gauge” from which to monitor 71% of Earth’s surface. By using ocean observations, we show the “rich get richer” mechanism is already operating, with fresh regions becoming fresher and salty regions

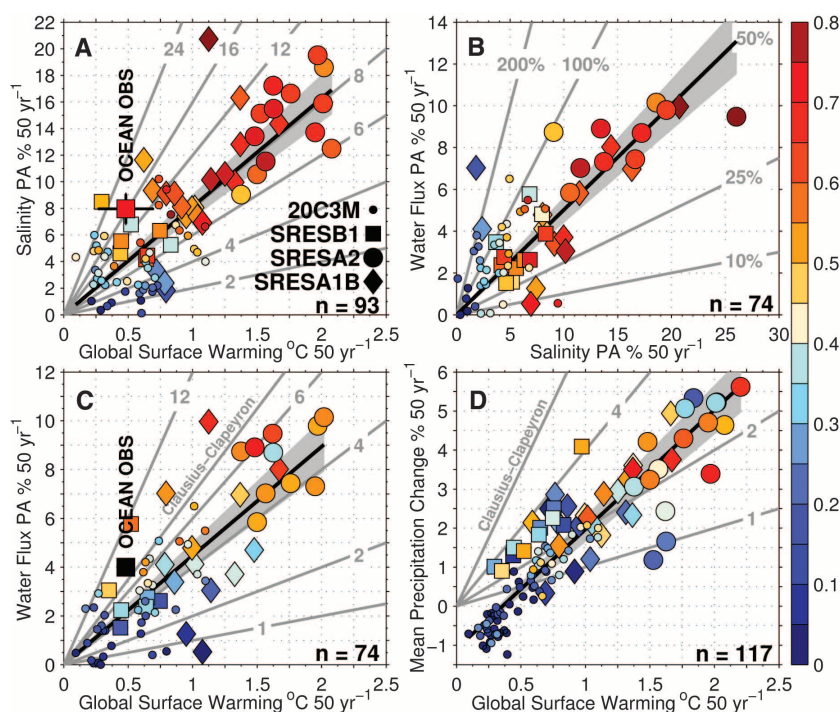


Fig. 2. PA and PCs from the available CMIP3 simulations compared with new observational estimates. The number of individual simulations that have been analyzed for each variable is noted in the bottom right-hand corner of each panel. (A) The surface salinity PA (y axis) versus the corresponding global ΔT_a (x axis); colors are the salinity PC. (B) Water flux (E-P; y axis) PA versus surface salinity PA (x axis); colors are the salinity PC. (C) Water flux (E-P; y axis) PA versus global ΔT_a (x axis); colors are the E-P PC. (D) Global spatial average precipitation change, rather than PA (ΔP ; y axis) versus global ΔT_a (x axis); colors are the precipitation PC. Gray lines express constant proportional change. Gray shading [99% confidence interval (C.I.)] bounds the PC-weighted linear best fit to the model ensemble for a line intersecting 0 [y axis in (A) to (C)] and -1.1 [y axis in (D)] in black. The 20th-century (20C3M; 1950–2000) simulations are presented in small circles, and the three 21st-century projected scenarios (SRES; 2050–2099) are shown as squares for B1, large circles for A2, and diamonds for A1B. All simulations have been de-drifted by using an appropriate preindustrial control simulation for the period 1900–2049 (supplementary text section 2). Observational estimates using a ΔT_a value from HadCRUT3 in (A) PA from this study are shown as the red square with black error bars showing the 99% significance level and in (C) as the black square with the CMIP3-scaled result based from (A) (see text).

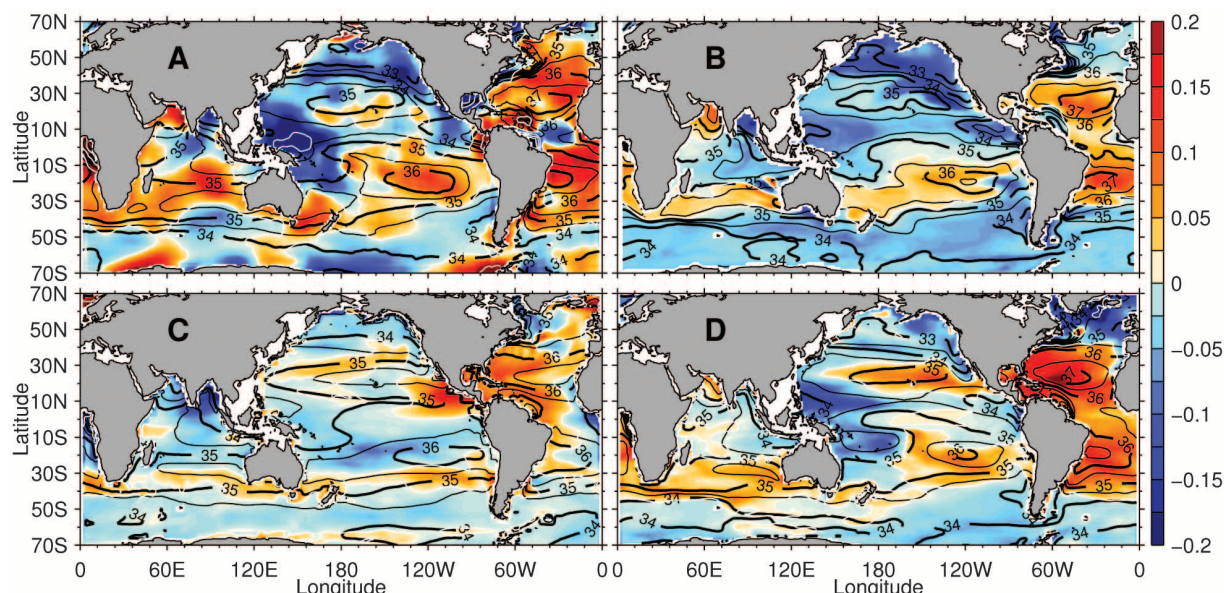


Fig. 3. Patterns of 50-year surface salinity changes (PSS-78 50 year⁻¹). (A) The 1950–2000 observational result (25). (B) From an ocean model forced with an idealized surface 5% E-P enhancement (50 year⁻¹; see text). (C) For an ensemble mean from 1950–2000 of the CMIP3 20C3M simulations that warm <0.5°C (24 simulations; see table S2). (D) For an en-

semble mean from 1950–2000 of the CMIP3 20C3M simulations that warm >0.5°C (26 simulations; see table S2). In each panel, the corresponding mean salinity from each representative data source is contoured in black, with thick lines every 1 (PSS-78) and thin lines every 0.5 (PSS-78).

saltier in response to observed warming. Our results support a water cycle intensification rate consistent with the CC relationship under fixed relative humidity. In a future GHG-forced 2° to 3°C warmer world (31), this implies a 16 to 24% amplification of the global water cycle will occur, nearly double the CMIP3 response.

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Supplementary Materials

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Supplementary Text
Figs. S1 to S9
Tables S1 to S3
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An Early-Branching Microbialite Cyanobacterium Forms Intracellular Carbonates

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Cyanobacteria have affected major geochemical cycles (carbon, nitrogen, and oxygen) on Earth for billions of years. In particular, they have played a major role in the formation of calcium carbonates (i.e., calcification), which has been considered to be an extracellular process. We identified a cyanobacterium in modern microbialites in Lake Alchichica (Mexico) that forms intracellular amorphous calcium-magnesium-strontium-barium carbonate inclusions about 270 nanometers in average diameter, revealing an unexplored pathway for calcification. Phylogenetic analyses place this cyanobacterium within the deeply divergent order Gloeobacterales. The chemical composition and structure of the intracellular precipitates suggest some level of cellular control on the biomineralization process. This discovery expands the diversity of organisms capable of forming amorphous calcium carbonates.

Cyanobacteria constitute a phylogenetically diverse group of bacteria that can carry out oxygenic photosynthesis (1). These cosmopolitan microorganisms occupy a wide array of terrestrial, marine, and freshwater habitats. They are major modern primary producers, as exemplified by the two marine cyanobacterial genera *Prochlorococcus* and *Synechococcus*, which are responsible for 25% of global photosynthesis (2). In addition to their evolutionary importance as ancestors of chloroplasts in photosynthetic eukaryotes (1), cyanobacteria have an extensive fossil record. Calcified cyanobacteria commonly occur since the base of the Cambrian, the earliest undisputed occurrence being *Girvanella* at 700 million years ago (3). Moreover, the occurrence of massive fossil stromatolites likely traces their emergence back to at least 2.7 billion years ago (4). Since then, they have played a major role in the carbon cycle by converting CO₂ into organic carbon and carbonates (2) and incidentally enriching the atmosphere in oxygen (5).

Carbonate precipitation by some cyanobacteria results from their photosynthetic activity (6, 7). Photosynthesis locally increases the concentration of CO₃²⁻ through the disproportionation of bicarbonate to carbonate and CO₂, which is fixed by ribulose-1,5-bisphosphate

carboxylase-oxygenase (RuBisCO). The export of alkalinity from the intracellular to the extracellular medium involves a mechanism that is still poorly documented. This export process raises the saturation index of the surrounding microenvironment for carbonate minerals, leading to mineral precipitation if free cations (e.g., Ca²⁺) and nucleation sites are available (6). In all cases described so far [e.g., (2, 8)], carbonate mineral precipitation by cyanobacteria is extracellular. As a result, the chemical composition of precipitates depends essentially on the surrounding environmental conditions, with little control by the cell (9). Calcification efficiency varies among different species as a function of extracellular surface properties, including exopolymer composition (10) or varying physiological states of the cell (11, 12). However, cyanobacterial calcification has been studied only in a few model species; thus, our knowledge of the mechanisms involved remains limited to a narrow phylogenetic window.

We studied biofilms dominated by cyanobacteria derived from modern microbialites collected in the highly alkaline (pH ~8.9) Lake Alchichica, Mexico (13). Previous studies have detected a large diversity of bacteria and microbial eukaryotes in these samples, plus a minor archaeal component (14, 15). Alchichica microbialites are mostly composed of hydromagnesite [Mg₅(CO₃)₄(OH)₂·4(H₂O)] and aragonite (CaCO₃). In the course of long-term studies of Alchichica microbialites maintained in laboratory aquaria, we observed the conspicuous development of phototrophic biofilms on aquarium walls (fig. S1). The biofilms were dominated by cyanobacteria of different sizes and morphotypes (13), also recognizable by confocal laser scanning microscopy (CLSM) according to their red autofluorescence (using a green excitation wavelength). Among these different morphotypes, one relatively small, rod-shaped, unicellular morphotype was particularly abundant (fig. S1). These cells measured

3.9 ± 0.6 μm in length and 1.1 ± 0.1 μm in width and exhibited a granular cytoplasm (Fig. 1, A and B, and table S1). Autofluorescence emission spectra measured on individual cells showed the presence of chlorophyll and phycocyanin, supporting their identification as cyanobacteria (fig. S2). Scanning electron microscopy (SEM) revealed spherical intracellular granules in this morphotype with average diameter of 270 ± 44 nm (Fig. 1, C to I, and table S1). The average number of inclusions per cell was 21 ± 5. These inclusions were very bright in backscattered electron and secondary electron modes, suggesting that they contained elements of high atomic number. Their chemical composition was determined by energy-dispersive x-ray spectroscopy (EDXS; Fig. 2A). Inclusions contained Ca, Mg, Ba, and Sr as major elements with atomic ratios Ca/Mg = 2.88, Ba/Mg = 1.11, and Sr/Mg = 0.42 (table S2). These ratios were markedly different from those measured in the solution; relative to the medium where cells grew, Ba/Ca and Sr/Ca atomic ratios in the inclusions were higher by factors of 1370 and 86, respectively (tables S2 and S3).

We investigated the chemical composition and structure of the Ca-Mg-Sr-Ba inclusions with the use of transmission electron microscopy (TEM) and scanning transmission x-ray microscopy (STXM) (13). STXM allows imaging and acquisition of x-ray absorption near-edge structure (XANES) spectra at high spectral (~0.1 eV) and spatial (~25 nm) resolution. A peak at 290.3 eV in the XANES spectra measured at the C K-edge on intracellular inclusions (Fig. 2C and table S4) was attributed unambiguously to 1s-π* electronic transitions in carbonate groups (16). Selected-area electron diffraction patterns obtained by TEM on these inclusions suggested that they are amorphous (Fig. 2B). XANES spectra at the Ca L_{2,3} edges are indicative of the local structure around Ca and can be used to characterize poorly organized phases [e.g., (16, 17)]. Spectra measured on intracellular inclusions were different from spectra of common reference Ca carbonates such as aragonite, calcite, or Ca-substituted strontianite, but they were very similar to the spectrum of benstonite, a Mg-Ca-Sr-Ba carbonate (Fig. 2D and table S5). The stoichiometric formula of the intracellular inclusions can thus be written as (Sr₁Ba_{2.7}Mg_{1.4}Ca_{0.9})Ca₆Mg(CO₃)₁₃, following that of benstonite and in agreement with EDXS analyses. Hence, these intracellular inclusions are composed of an unusual benstonite-like phase with no long-range order and an unusual stoichiometry. In total, inclusions occupied ~6% of the total cell volume, raising the total density of the cells by 12% (tables S6 and S7).

We enriched these cyanobacteria in culture by inoculating modified BG11 medium with cells smaller than 3 μm from disrupted biofilms. CLSM and SEM observations showed that six enrichment cultures (out of 96) contained this single cyanobacterial morphotype with intracellular inclusions—that is, with no additional cyanobacterial morphotype as detected by microscopy

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(fig. S3). This was further confirmed by amplification and sequencing of 16S ribosomal RNA (rRNA) genes using cyanobacterial-specific primers, which provided 100% identical sequences within and among the six enrichments (table S8). Phylogenetic analysis showed that this strain was a member of the basal cyanobacterial order Gloeobacterales (Fig. 3).

The sequences obtained from the enrichment cultures were almost identical (99.7% sequence identity) to AQ1_1_1C_35 (CyanoOTU02) (13), an environmental sequence abundantly retrieved from aquarium microbialites (up to 20% of cyanobacterial sequences) (15). This indicates that this lineage is an important cyanobacterial component of actively growing microbialites, being

able to disperse and form neighboring biofilms on aquarium walls. Because it is phylogenetically very distant from *Gloeobacter*, the only genus described within Gloeobacterales, we propose the following status: order Gloeobacterales, *Candidatus Gloeomargarita lithophora* gen. et sp. nov. (13).

The amorphous intracellular bioprecipitates are structurally and chemically different from the well-crystallized phases forming in the extracellular solution (i.e., aragonite and hydro-magnesite) (tables S2 and S3). Incorporation of Sr and Ba in carbonates has implications for Sr/Ca and Ba/Ca ratios in marine carbonates, which are frequently used as paleoenvironmental proxies (18). Such proxies rely on the assumption

that these ratios are approximately the same (or slightly lower) in the solid phases as in the solution. In *Candidatus G. lithophora* intracellular inclusions, these ratios are much higher in the solid phase and are similar to what has been found in some algae (19). Such an enrichment in Sr and Ba might involve active import systems specific for Sr^{2+} and Ba^{2+} and/or export systems extruding specifically Ca^{2+} and maintaining low cytoplasmic Ca^{2+} levels (20). The amorphous structure of the inclusions is somewhat similar to relatively unstable amorphous calcium carbonates (ACCs) observed in biological, mostly eukaryotic systems (21). Consistent with what has often been observed in these ACC-containing systems (21), the presence of organic macromolecules may

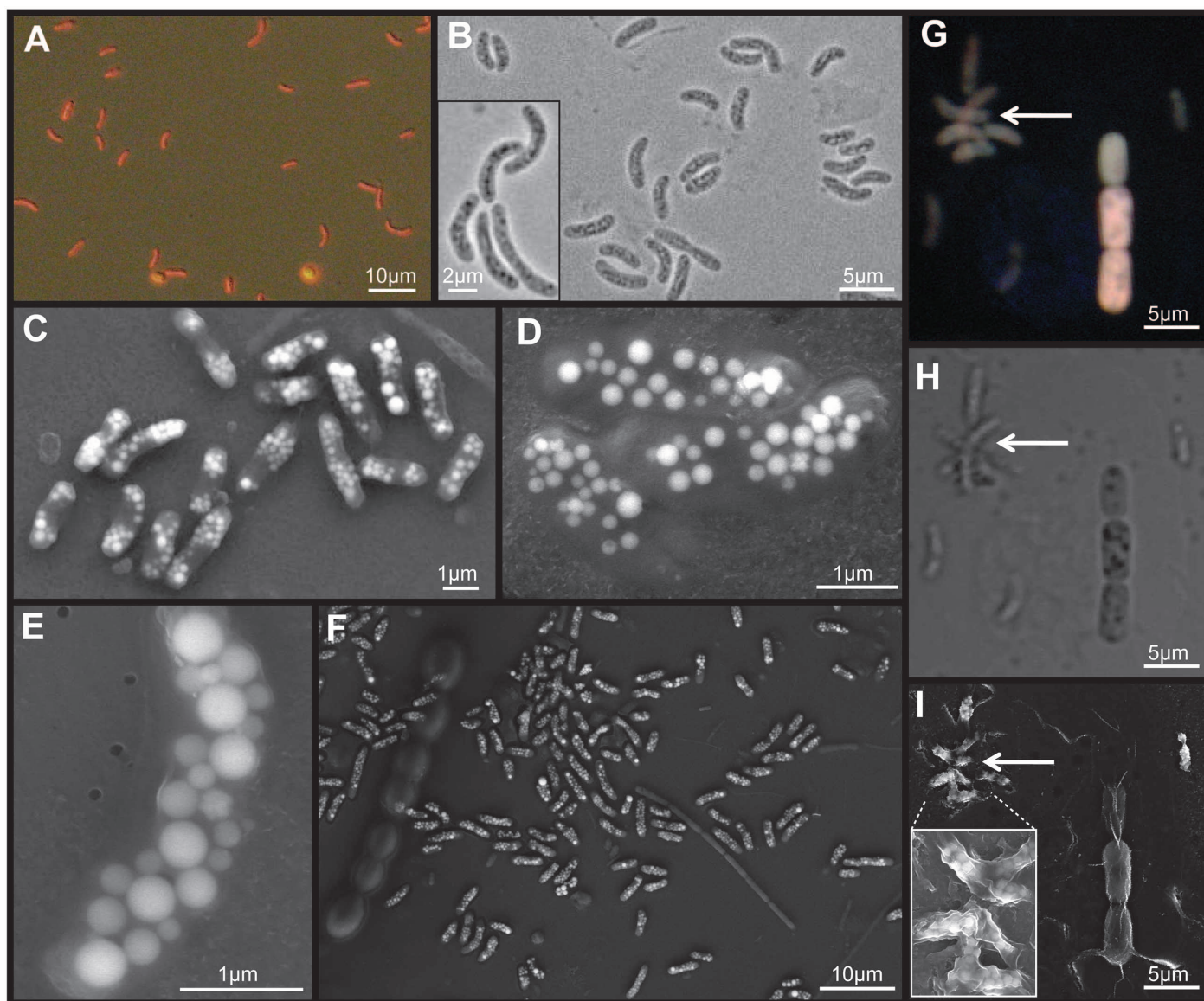


Fig. 1. Visualization of cyanobacterial cells forming intracellular mineral inclusions. (A) Composite epifluorescence and optical microscopy images showing rod-shaped autofluorescent cyanobacteria cells. (B) Optical image shows intracellular inclusions in cyanobacterial cells (higher magnification in inset). (C to F) SEM images (secondary electron mode) showing cells of the

same morphotype, which systematically contain bright intracellular inclusions. (G to I) CSLM (G), phase contrast (H), and SEM (I) images of exactly the same area, showing that autofluorescent cells observed by CSLM contain the bright inclusions observed by SEM (inset). A different, larger, and inclusion-deprived cyanobacterial morphotype appears on the right.

stabilize the intracellular amorphous carbonates observed in *Candidatus G. lithophora*. Diverse local structures were recently suggested for these phases lacking long-range order (22). The Mg-, Ca-, Sr-, and Ba-containing inclusions expand the known diversity of ACC compositions.

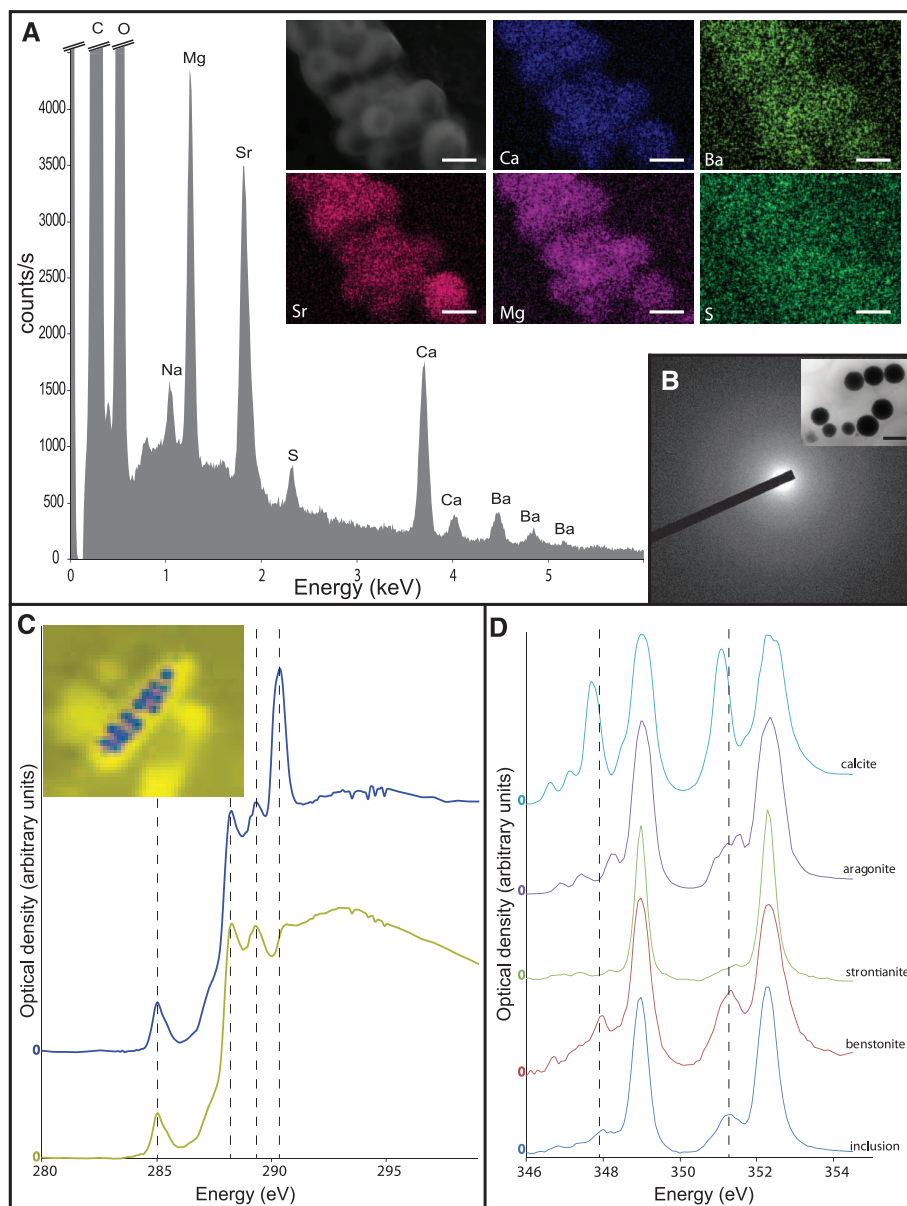
Intracellular carbonate precipitation in this cyanobacterium likely influences its biology and ecology. First, by changing average cell density, these precipitates must alter cell buoyancy. The conspicuous occurrence of *Candidatus G. lithophora* in aquarium wall biofilms implies that a dispersive planktonic phase exists that allows the colonization of surrounding surfaces. Therefore, whereas gas vesicles function as flotation devices in planktonic cyanobacteria and other prokaryotes (23), intracellular carbonates might serve as ballast—an adaptation to a benthic mode of life. Second, the formation of intracellular carbonates implies that the alkalin-

ity excess produced by cyanobacteria during photosynthetic carbon fixation, a process taking place in carboxysomes, is not always exported extracellularly but can be trapped within amorphous carbonate inclusions. Whether carboxysomes could also provide nucleation centers for carbonate precipitation remains an open question. Carbonate precipitation could act as a pH buffering system leading to an efficient carbon-concentrating mechanism and hence a high photosynthesis rate (3).

Because intracellular biomineralization occurs in a phylogenetically basal lineage of cyanobacteria that has retained ancestral characteristics (24) and because the alkalinity export may require the evolution of specific machinery, it might be argued that this calcification mode was performed by some ancestral cyanobacteria. Gloeobacterales is the only cyanobacterial order branching out before the chloroplast clade in

molecular phylogenies of conserved gene markers (Fig. 3) (25). Not only *Gloeobacter violaceus*, isolated from limestone biofilms (24), but also most environmental sequences forming the large Gloeobacterales clade have been retrieved from rock-associated biofilms, stromatolites, or thermophilic microbial mats (Fig. 3). This association indicates a preference for these types of substrates and suggests that Gloeobacterales ancestors may have also exhibited the capacity to form biofilms in moderately hot and/or calcifying environments. Intracellularly biomineralizing cyanobacteria would not be prone to the formation of microfossils, in contrast to cyanobacteria inducing extracellular precipitation of carbonates within sheaths (3, 6). As a result, microbialites dominated by intracellularly biomineralizing cyanobacteria might be devoid of microfossils. Alternatively, if they can be discriminated from abiotically formed phases (26), traces of this

Fig. 2. Chemical and mineralogical characterization of intracellular inclusions. **(A)** SEM-EDXS spectrum and associated chemical maps obtained on inclusions (scale bars, 300 nm). Only Ca, Mg, Sr, and Ba are specifically associated with the inclusions, whereas S is distributed over the whole cells. **(B)** Selected-area electron diffraction pattern of an intracellular inclusion (inset; scale bar, 300 nm) showing a broad ring characteristic of an amorphous phase. **(C)** Average STXM-based XANES spectra with associated map (inset) measured at the C K-edge on areas showing only cellular material (yellow) and areas containing inclusions (blue). Spectra are shifted with respect to each other along the y axis for clarity; each spectrum has a different origin in y. Peak labels are reported in table S4. **(D)** STXM-based XANES spectrum measured at the Ca L_{2,3}-edges on intracellular inclusions. This spectrum is compared with spectra of reference calcite, aragonite, strontianite, and benstonite. The XANES spectrum of the intracellular inclusions matches that of benstonite in peak energy position and relative peak intensities. Peak positions are reported in table S5.



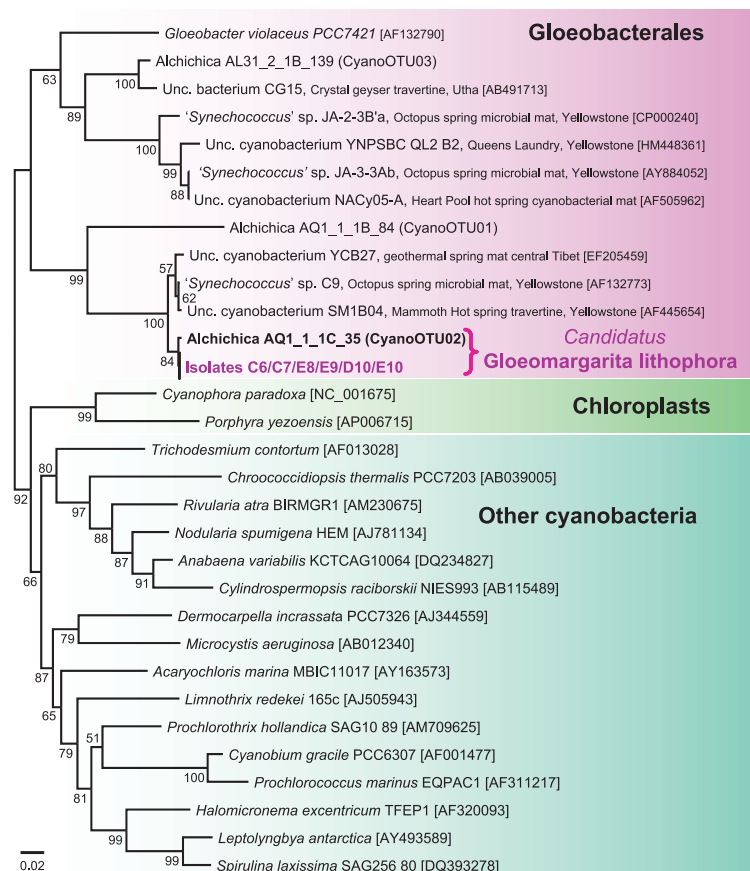


Fig. 3. Phylogenetic position of the intracellularly calcifying cyanobacterium *Candidatus Gloeomargarita lithophora*. A maximum likelihood phylogenetic tree of cyanobacterial small subunit ribosomal DNAs was constructed from 1191 unambiguously aligned positions. Sequences obtained from various enrichment cultures were 100% identical (pink) and very closely related to the environmental sequence Alchichica AQ1_1_1C_35 CyanoOTU02, abundantly retrieved from cultured microbialites. Numbers at nodes indicate bootstrap values. The scale bar indicates the number of substitutions per site for a unit branch length.

biomineralization activity may be sought in the form of Ba- and Sr-rich carbonates. These Ba- and Sr-rich phases therefore add to the list of potential mineral biosignatures in the fossil record.

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Supplementary Materials

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Materials and Methods

Supplementary Text

Figs. S1 to S4

Tables S1 to S8

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In Situ Evolutionary Rate Measurements Show Ecological Success of Recently Emerged Bacterial Hybrids

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Few data are available on how quickly free-living microorganisms evolve. We analyzed biofilms collected from a well-defined acid mine drainage system over 9 years to investigate the processes and determine rates of bacterial evolution directly in the environment. Population metagenomic analyses of the dominant primary producer yielded the nucleotide substitution rate, which we used to show that proliferation of a series of recombinant bacterial strains occurred over the past few decades. The ecological success of hybrid bacterial types highlights the role of evolutionary processes in rapid adaptation within natural microbial communities.

Microbial communities, which drive Earth's geochemical cycles (*1*), can rapidly respond to change, but the proportion of

this response that can be attributed to evolutionary processes, rather than species composition or gene expression shifts, remains an unresolved

question (2). Most evolutionary rate estimates are available for nucleotide substitution rates and derive from laboratory measurements (3, 4). It is difficult to know how relevant these rates are for geochemical environments, because studies on natural populations have been restricted to pathogens (5–7) and endosymbionts (8).

Our approach to measuring evolutionary rates for free-living bacterial populations involved tracking genome change in a time series of natural microbial community samples. Few systems are suitable for such a study; requirements include the availability of discrete, well-defined, and relatively homogeneous microbial community samples from a location where a reproducible community forms over time, with very restricted microbial input from other regions. The microbial communities catalyzing the formation of acid mine drainage (metal-rich, low-pH solutions) in the Richmond Mine (Iron Mountain, CA) provide such a system. Biofilms that develop at the air-solution interface on standing pools and slow-flowing underground streams have been used for more than a decade to study evolutionary processes and ecological complexity in nature (9). Key to the success of this environment as a model system has been its low species richness, which enables detailed culture-independent community genomics analyses of the genetic structure and dynamics of natural populations (10–13). Most biofilms within this system are dominated by the chemolithoautotrophic *Leptospirillum* group II. Previous metagenomic studies on two biofilms led to the reconstruction of genomes for *Leptospirillum* group II, types I and VI, the genes of which share ~94% average nucleotide identity (10, 13, 14). Inferences based on proteomics data indicate that large-scale homologous recombination events occurred between two parental *Leptospirillum* group II types, resulting in six recombinant (hybrid) genotypes (14, 15). One genotypic group, comprising types II through VI, predominates during initial colonization, whereas type I becomes abundant in later successional stages (16).

We have collected biofilms within the Richmond Mine over approximately 9 years. Sampling required field expeditions to locations where ambient conditions are often close to the limit of human endurance (e.g., 100% humidity, ~48°C, with depressed O₂ levels) at sites only accessible at times of the year when flow rates are low and danger from underground collapse is minimized. Working within these constraints, we collected microbial biofilms (both time series and spatially resolved) from six underground locations between March 2002 and December 2010 at intervals of 3 weeks to more than 1 year (Fig. 1, table S1, and

figs. S1 and S2) (17). We selected 13 samples from the C75 location, a site indicated by proteomics-based inferences to be typically dominated by the *Leptospirillum* group II, type III genotype (15). This allowed us to analyze single-nucleotide substitution accumulation over 5 years, and thereby to estimate the substitution rate. On the basis of the same proteomics-based inferences, we also selected nine biofilms that provided multiple samplings of most of the other *Leptospirillum* group II genotypes (types I, III, IV, V, and VI). Together with the two sequence data sets from which the type I and type VI reference genomes had been reconstructed, this allowed us to reconstruct a lineage history (fig. S1).

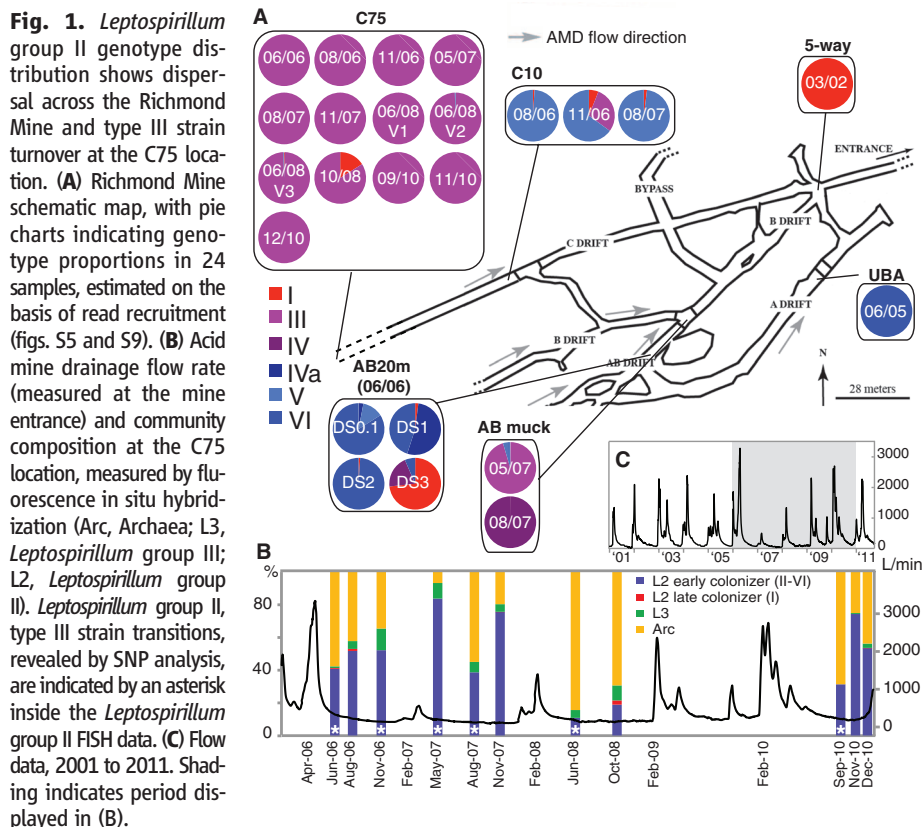
We generated community genomic data sets comprising ~63 billion base pairs of sequence from 24 samples, each of which contained ~10⁸ *Leptospirillum* group II cells (10). Using sequences obtained from the first five C75 location biofilms, for which longer read lengths were available, we reconstructed a type III genome de novo (17). The genome is indeed a recombinant hybrid of the previously reconstructed type I and type VI genomes, as deduced from proteomic analysis (15). All identified recombination points were located within genes (table S2 and fig. S3). All *Leptospirillum* group II sequences were accounted for (fig. S4) (17), excluding the possibility of other genotypes in these five samples.

The sequencing reads from each of the 13 C75 samples collected over 5 years were recruited to the previously reconstructed *Leptospirillum* group II, type I and type VI genomes (13, 18) to identify

the recombinant block structure of the genome at each time point at this location. Only alignment of reads to the sequence with the highest similarity was permitted. Twelve biofilms contained only the type III genotype (>99% of cells); the remaining one contained ~84% type III and ~16% type I genotype (Fig. 1 and figs. S5 and S6A). Because 1.7 million to 8 million Illumina sequencing reads were obtained per sample (table S1), and because each read is an independent measurement of population-level sequence variation, we could unambiguously identify high-frequency single-nucleotide polymorphisms (SNPs) (>90% of all reads).

Nucleotide by nucleotide, we compared the type I-like segments of the type III genome to the type I genome sampled in 2002. Similarly, type VI-like regions of the type III genome were compared to the type VI genome sampled in 2005. In total, relative to the reference sequences, 64 mutations were fixed in all populations sampled at the C75 location (fig. S7A).

We then tracked genome change at the C75 location between August 2006 and December 2010. In the 11 C75 samples taken after August 2006, we identified up to six additional high-frequency substitutions relative to the 2006 type III genome (fig. S7A). Notably, the number of high-frequency mutations increased over time (fig. S8, $R^2 = 0.78$). Using this information, we calculated a rate of 1.4×10^{-9} (SE, $\pm 0.2 \times 10^{-9}$) substitutions per nucleotide per generation (table S3). This number is in the higher range of previously reported bacterial genome-wide short-term substitution rates [7.2×10^{-11} to 4.0×10^{-9} substitutions per



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nucleotide per generation (4, 7, 8)]. It is similar to the rate of 1.3×10^{-9} substitutions per nucleotide per generation predicted for the type III genome size using the universal mutations-per-genome rate suggested by Drake (3). Although the highest rates have been reported for endosymbionts and pathogens, which have small effective population sizes, correspondence with Drake's prediction may indicate that genetic drift accelerated by population bottlenecks is not the main factor affecting the substitution rate in *Leptospirillum* group II.

To assess potential biases in the measured substitution rate introduced by spatial variation within the contiguous biofilms covering the C75 acid mine drainage pool, we sampled three loca-

tions ~1 m apart in June 2008. Samples 1 and 3 were dominated by the same genotype (with four SNPs relative to the type III genome), whereas sample 2 was dominated by a variant of this genotype lacking one high-frequency polymorphism (fig. S7A). The three samples also had only 93 (± 8) replicated SNPs at frequency greater than 0.05 (average frequency 0.08). The low levels of variation, both within populations and across space, provide confidence that nucleotide changes in populations at the C75 location over time can be used to calculate the substitution rate.

Read recruitment of community genomic data from sites located across the underground system (five-way, UBA, C75, C10, AB muck, and AB20 locations) to types I and VI yielded

six genotypes (Fig. 1 and fig. S9): type I, type VI, and four other types (III, IV, IVa, and V) that each consist of a mosaic of type I and type VI genome blocks tens to hundreds of kilobases in length (Fig. 2, A and B, and figs. S6B and S9). This again confirmed previous strain-resolved proteomic analysis-based inferences (14, 15). The presence of exactly the same transition points between the recombinant blocks in each of the genotypes in every sample indicates that each major recombination event occurred in a single cell, whose descendants rose to fixation (Fig. 2). Each genotype's fixed mutations were identified by read recruitment to type III (Fig. 3 and fig. S7B) (17) and were used to construct a phylogenetic tree (Fig. 2C). Because read recruitment was performed

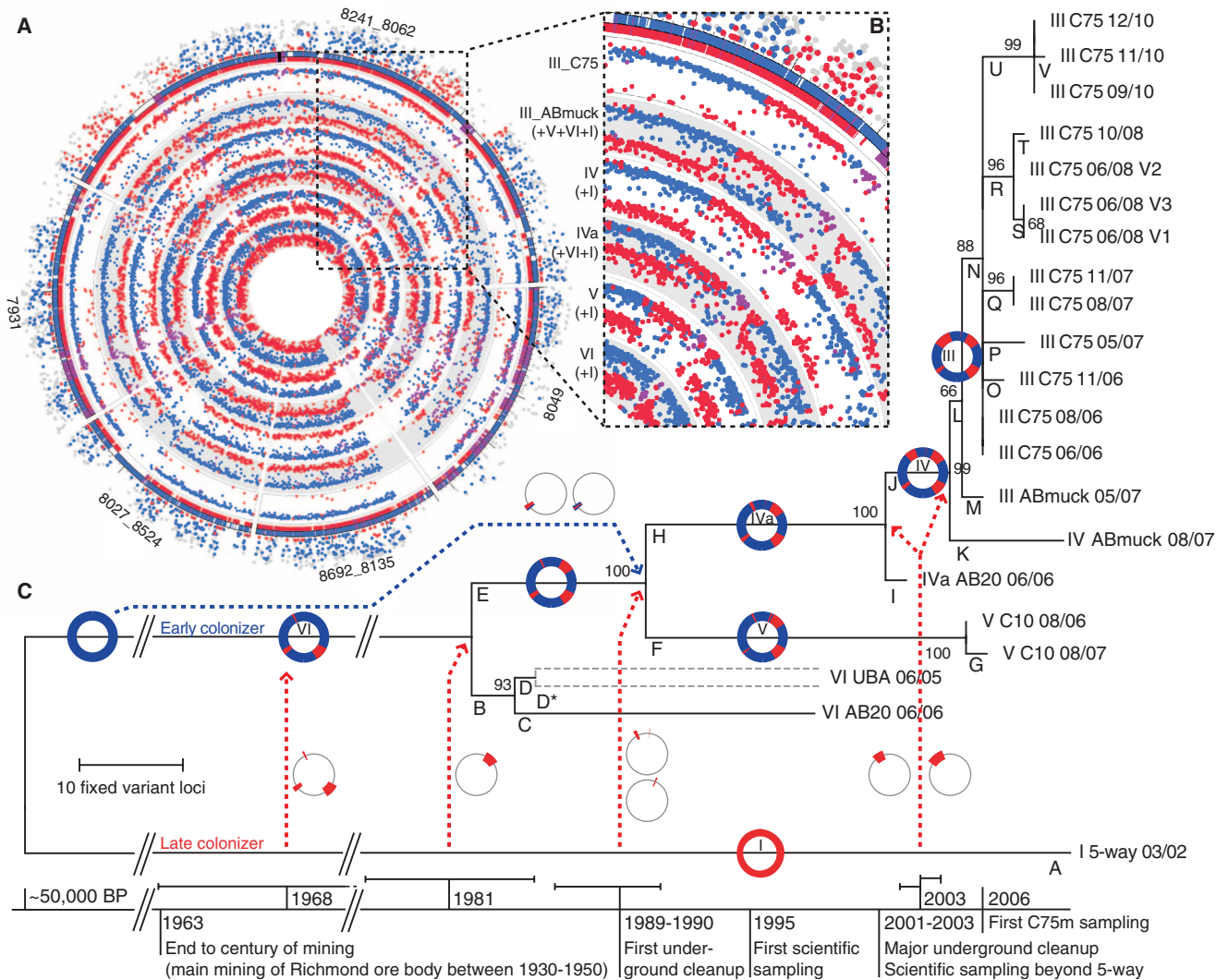


Fig. 2. Reconstruction of the recent evolutionary history of *Leptospirillum* group II. (A) The outer circle plots the counts of peptides unique to the type I (red) and type VI (blue) reference genomes at each protein locus (dots) for the C75 type III population [proteomics-inferred genotyping (PIGT); data from (15)]. Inner circles show Illumina read recruitment to the reference genomes, revealing the prevalence of type III, IV, IVa, V, and VI recombinants. (B) Correspondence of PIGT and recruitment data, highlighting the identical recombination point in genotypes III to V. (C) Evolutionary history of the sampled genotypes, based on

the variant loci (table S7), inferred using the maximum parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. Dotted arrows indicate recombination events; circle schematics represent the regions affected. The timeline indicates the calculated time ranges of recombination events (table S3) as well as historical events (fig. S2) (18, 21). Branch D* is presented as two strains, each assigned half the total number of UBA 06/05 SNPs, because low incidence of SNPs precluded their linkage. BP, years before the present.

relative to the type III genome, positions within type I-like regions not shared by type IV, V, or VI genotypes were designated as gaps for tree construction, and these gaps were treated as missing data in the calculation of tree branch length. Notably, the tree topology indicates that the larger the fraction of type I-like sequence recombined into the type VI background, the more recently the specific genotype emerged (Fig. 2C).

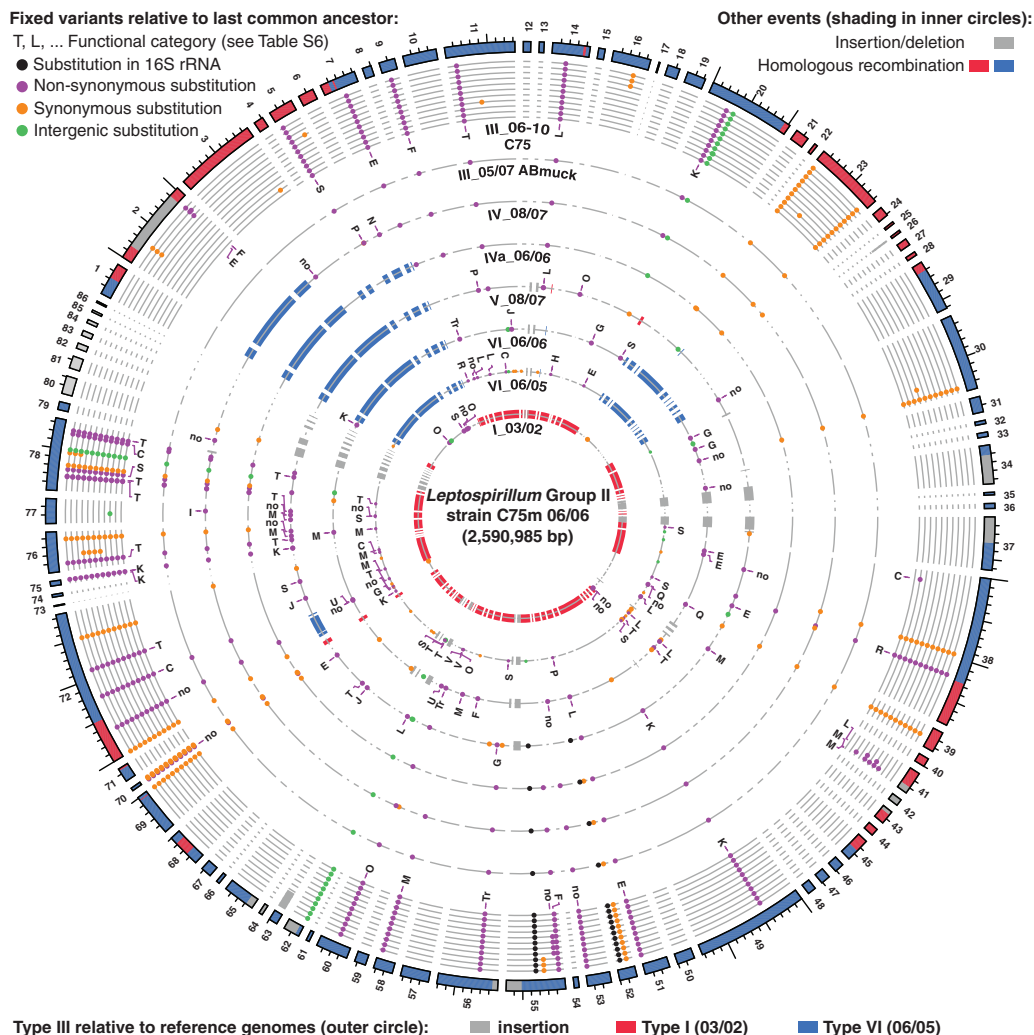
Using the substitution rate calculated from the C75 time series and assuming equal generation times for the different genotypes, we estimated that the times to coalescence for the suite of recombinant genotypes ranged between 2 and 44 years (Fig. 2 and table S4). The causes of the success of these hybrid genotypes are hard to establish. In Bacteria and Archaea, little is known about the importance of hybridization in adaptation to change. However, one recent study hypothesized that agricultural practices led to proliferation of hybrids of *Campylobacter jejuni* and *Campylobacter coli* formed by homologous recombination (19). In Eukarya, hybrids are often reported to have little ecological success (20). However, increased fitness relative to parental types has been observed when the adaptive landscape changes as a result

of natural- or human-induced environmental alterations (21, 22). The Richmond Mine site is subject to both natural (seasonal and year-to-year variation; Fig. 1C) and human perturbations (fig. S2) (17), a subset of which may have provided opportunity for hybrid proliferation.

Although we cannot connect specific events to emergence of the hybrid genotypes, we can evaluate the role of neutral versus selection-based evolutionary processes in lineage divergence. For example, extreme population bottlenecks such as flushing events that remove biofilms during the rainy season may purge diversity independent of the fitness of particular strains. However, there are several lines of evidence indicating that hybrid genotypes arose as the result of selective processes. We detected evidence for positive selection (McDonald-Kreitman test, $P < 0.05$) for mutations specific to one type VI population (branch C; see Fig. 2), type V (branch F), and the ancestor of types III to V (branch E) (table S5). Selection was also supported by the disproportionately high number of signal transduction genes (Fig. 3, functional category T) and transcriptional regulation genes (Fig. 3, category K), global regulators in particular, that were affected by

fixed mutations (Fig. 2, branches E, F, D*, and H; see also tables S5 and S6). Previous observations in laboratory settings (23–25) and a natural system (16) similarly identified evolution of gene expression as an important factor underpinning early ecological divergence. Finally, an earlier study analyzing the distribution of the different *Leptospirillum* group II genotypes in the context of geochemical conditions indicated selection among type I through type VI recombinants (15). Evidence for selection also emerged from the current study, where two sites located ~140 m apart along a single flow path were compared (C10 and C75). The type III population, which dominated all upstream C75 biofilms, was found in only one of three biofilms sampled at the same time at the downstream C10 location. Even when the type III population was present at both sites, the genomes were different (Fig. 1 and fig. S10). This result suggests selection between genotypes that differ by only a few nucleotides—a finding consistent with prior laboratory studies showing increased fitness due to adaptive SNPs (4, 25). It also indicates that lack of diversity is not the explanation for dominance of many biofilms by a single genotype.

Fig. 3. Overview of the *Leptospirillum* group II high-frequency variants, based on read recruitment to the C75 June 2006 type III genotype. Dots indicate variant nucleotides relative to the last common ancestor of types III to VI. Functional categories (table S6) are indicated only for nonsynonymous substitutions and only for the first occurrence, starting from the most recent genotype (outside circle). Shading in inner circles indicates regions that were excluded for SNP analysis in a particular genotype because of recombination or indel events.



Our population genomic analyses provided data on the accumulation of genome change in free-living populations that underpin geochemical cycles. Application of these rates revealed that a series of important divergence events occurred over a time scale of years to decades within the natural model system of the Richmond Mine. We attribute these to major selection episodes, although we cannot conclude whether they were mediated by natural or human factors. Rapid adaptive evolution may have assisted in the maintenance of *Leptospirillum* group II as both the dominant primary producer and most active iron oxidizer responsible for acid mine drainage formation. Our results contribute to the development of a predictive understanding of how microbial systems respond to both natural and anthropogenic change (2).

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Supplementary Materials

www.sciencemag.org/cgi/content/full/336/6080/462/DC1
Materials and Methods
Figs. S1 to S11
Tables S1 to S7
References (26–48)

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Origins and Genetic Legacy of Neolithic Farmers and Hunter-Gatherers in Europe

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The farming way of life originated in the Near East some 11,000 years ago and had reached most of the European continent 5000 years later. However, the impact of the agricultural revolution on demography and patterns of genomic variation in Europe remains unknown. We obtained 249 million base pairs of genomic DNA from ~5000-year-old remains of three hunter-gatherers and one farmer excavated in Scandinavia and find that the farmer is genetically most similar to extant southern Europeans, contrasting sharply to the hunter-gatherers, whose distinct genetic signature is most similar to that of extant northern Europeans. Our results suggest that migration from southern Europe catalyzed the spread of agriculture and that admixture in the wake of this expansion eventually shaped the genomic landscape of modern-day Europe.

The transition from the hunter-gatherer lifestyle to a sedentary farming economy spread throughout Europe from the south-

east, starting ~8400 years before the present (yr B.P.) (1). Early population genetic studies argued that clines in genetic variation within Europe favored a model in which this expansion occurred in concert with substantial replacement of resident hunter-gatherer populations (2–4), contradicting models emphasizing the culturally mediated spread of farming economy (5). Current results from extant populations are inconclusive (4, 6); however, ancient DNA analyses (7–10) present tentative evidence for population replacement but suffer from uncertainties associated with single-locus studies of the Y chromosome or the mitochondrion. Population genomic analysis of ancient human remains (6, 11) shows

promise, but poses technical difficulties due to low DNA yield and the risk of present-day human contamination (6). In addition, geographical and temporal differences between ancient samples make observations of genetic differentiation difficult to interpret. Thus, more robust interpretations of ancient DNA might be gained by analyzing samples from cultural complexes occurring in the same region and during the same time period. In this study, we focused on the Neolithic era of northern Europe, where the relatively late arrival of farming (~6000 yr B.P.) was followed by more than 1000 years of coexistence between hunter-gatherer and farming cultures (12).

We obtained genomic DNA sequences from three samples (Ajr52, Ajv70, and Ire8) from a hunter-gatherer context [“Neolithic hunter-gatherers” associated with the Pitted Ware Culture (PWC)], one sample from a farming context [“Neolithic farmer” associated with the Funnel Beaker Culture or *Trichterbecher kultur* (TRB)], and two animal remains as contamination controls. The human remains were chosen from a larger panel on the basis of their molecular preservation and previously yielded reproducible single-locus genetic data (8, 13, 14). The Neolithic farmer sample (Gök4) was excavated from a megalithic burial structure in Gökhem parish, Sweden, and has been directly ¹⁴C-dated to 4921 ± 50 calibrated yr B.P. (cal yr B.P.), similar to the age (5100 to 4900 cal yr B.P.) of the majority of other finds in the area (15). There were no indications from the burial context suggesting that Gök4 was different from other TRB individuals (15, 16), and strontium isotope analyses indicate that Gök4 was born less than 100 km from the

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megalithic structure, similar to all other analyzed TRB individuals from the area (17). The three Neolithic hunter-gatherer samples were excavated from burial grounds with single inhumation graves on the island of Gotland, Sweden, for which associated remains have been dated to 5300 to 4400 cal yr B.P. (16).

Indexed libraries were prepared from decontaminated DNA extracts (8, 13) in dedicated

ancient DNA facilities and sequenced with the Illumina GAIIX platform (16). The fraction of nonredundant reads that could be mapped (18) to the human genome in the two animal samples was 0.02 to 0.12%, which is substantially lower than the 2.4 to 6.3% found in the human samples (Table 1). Furthermore, the sequences from the human remains showed characteristic features of ancient DNA degradation, including

short fragment read length [average ~55 base pairs (bp)], nucleotide misincorporations, and an increased fraction of purines close to sequence read termini (19) (fig. S12). Moreover, the mitochondrial DNA (mtDNA) sequences assembled to ~4- to 14-fold coverage from our shotgun data (Table 1) matched results previously obtained by polymerase chain reaction-based sequencing (8). These lines of evidence

Table 1. Summary of ancient genomic sequence data from Neolithic individuals.

Sample	Archaeological context	Fraction of human sequence reads*	Human autosomal sequence reads†	Human autosomal sequence data (bp)‡	Genotyped SNPs			mtDNA genome assembly	
					HM3+ FINHM+ HGDPS§	FINHM+ POPRES§	1KGPomni§	Mean depth	mtDNA haplogroup
Ajv52	Neolithic hunter-gatherer	2.38%	1,029,854	59,563,626	7,531	4,189	23,123	14.0X	V
Ajv70	"	4.85%	1,659,553	97,210,801	12,193	6,824	39,014	13.8X	U4d/U4a2
Ire8	"	2.71%	601,416	26,843,368	3,279	1,905	10,273	9.8X	U4d
Gök4	Neolithic farmer	6.31%	1,208,835	65,535,732	7,712	4,413	24,311	4.2X	H

*Nonredundant reads mapping to the human genome in libraries amplified by Phusion polymerase. †Mapping quality ≥30, nonredundant autosomal data only. ‡Mapping quality ≥30, base quality ≥15, nonredundant autosomal data only. §See Fig. 1 legend and (16) for an explanation of abbreviations.

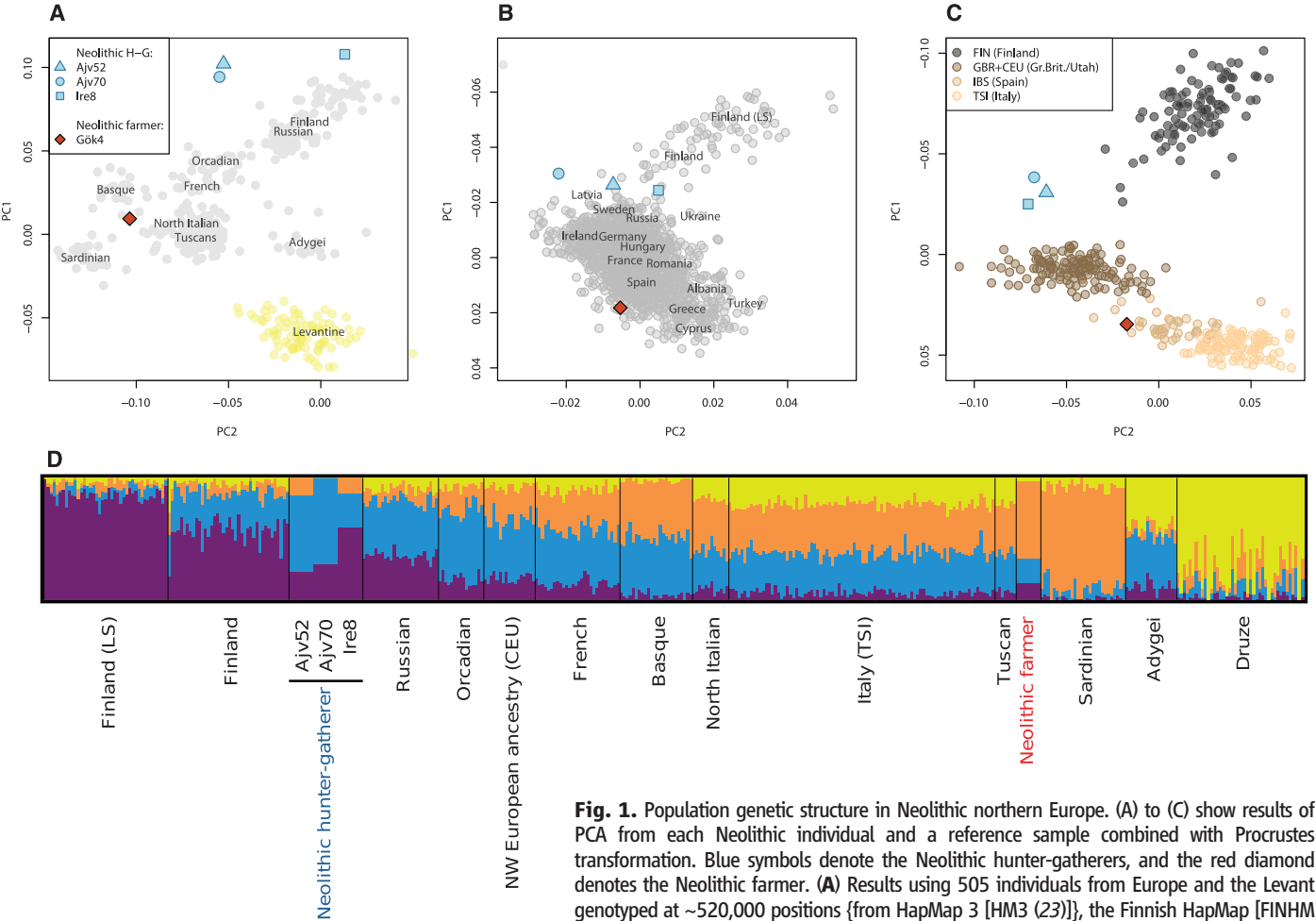


Fig. 1. Population genetic structure in Neolithic northern Europe. (A) to (C) show results of PCA from each Neolithic individual and a reference sample combined with Procrustes transformation. Blue symbols denote the Neolithic hunter-gatherers, and the red diamond denotes the Neolithic farmer. (A) Results using 505 individuals from Europe and the Levant genotyped at ~520,000 positions {from HapMap 3 [HM3 (23)], the Finnish HapMap [FINHM (22)], and the Human Genome Diversity Panel [HGD (21)] (16). Centroids are indicated with the population label. (B) Results using 1466 European individuals genotyped at ~280,000 positions {FINHM and the Population Reference Sample [POPRES (24)] (16). Selected centroids are indicated with the population label. (C) Results using 241 individuals from Europe genotyped at ~2.3 million positions {1000 Genomes Project [1KGPomni (25)] (16). (D) Population structure in Neolithic individuals and extant individuals from Europe and the Levant inferred by model-based clustering (25). Each individual is shown as a vertical line partitioned into colored components representing the inferred membership in four genetic clusters.

with the population label. (B) Results using 1466 European individuals genotyped at ~280,000 positions {FINHM and the Population Reference Sample [POPRES (24)] (16). Selected centroids are indicated with the population label. (C) Results using 241 individuals from Europe genotyped at ~2.3 million positions {1000 Genomes Project [1KGPomni (25)] (16). (D) Population structure in Neolithic individuals and extant individuals from Europe and the Levant inferred by model-based clustering (25). Each individual is shown as a vertical line partitioned into colored components representing the inferred membership in four genetic clusters.

point to the presence of substantial amounts of endogenous DNA. After quality filtering, we analyzed 249 million bp of autosomal sequence data and extracted genetic variants from the Neolithic individuals at previously identified single-nucleotide polymorphisms (SNPs) in various reference data sets (Table 1), excluding SNPs that could be affected by postmortem nucleotide misincorporation and randomly sampling a single haploid variant from both ancient and modern individuals (16).

Because the incomplete coverage causes little overlap between the typed SNPs in the different Neolithic individuals, we performed principal

component analysis (PCA) (20) separately for each Neolithic individual, together with a particular reference panel, and combined PC1 and PC2 loadings from each independent analysis, using a novel approach based on Procrustes transformation (16). We found that compared to a worldwide set of 1638 individuals (21–23), all four Neolithic individuals clustered within European variation (fig. S5). However, when the analysis was focused on 505 individuals of European and Levantine descent, the three Neolithic hunter-gatherers appeared largely outside the distribution of the modern sample but in the vicinity of Finnish and northern European indi-

viduals (Fig. 1A). In contrast, the Neolithic farmer clustered with southern Europeans but was differentiated from Levantine individuals. This general pattern persisted for a geographically broader reference data set of 1466 extant individuals of European ancestry (22, 24) (Fig. 1B), for a much larger number of markers from 241 individuals in the 1000 Genomes Project (25) (Fig. 1C), and using model-based clustering (26, 27) (Fig. 1D). Although all Neolithic individuals were excavated in Sweden, neither the Neolithic farmer nor the Neolithic hunter-gatherers appeared to cluster specifically within Swedish variation, a pattern that remained also for a larger sample of 1525 individuals from across Sweden (28) (figs. S9, S21, and S22).

Although several observations suggest that authentic ancient molecules were sequenced, it is possible that some degree of contamination from modern humans could be present in the data. To investigate whether contamination could influence our analyses, we partitioned the data on the basis of the fraction of cytosine deamination toward the ends of the sequence reads (Fig. 2A), a property that has been observed to be enriched in authentic ancient DNA but not in contaminating modern human molecules (19, 29, 30). We divided the data into sequences that had evidence of cytosine deamination in the first 10 bp of the read and those that did not. Examining the first principal component obtained for Neolithic individuals and extant individuals from Italy and Finland, combined by means of Procrustes transformation, we found a robust separation between the Neolithic hunter-gatherers and the Neolithic farmer in both the data with evidence of cytosine deamination and the data without (Fig. 2B).

To more closely investigate the genetic similarity of extant European populations (22, 24) to Neolithic humans, we determined for each SNP and each extant population the average frequency of the particular allele found in either

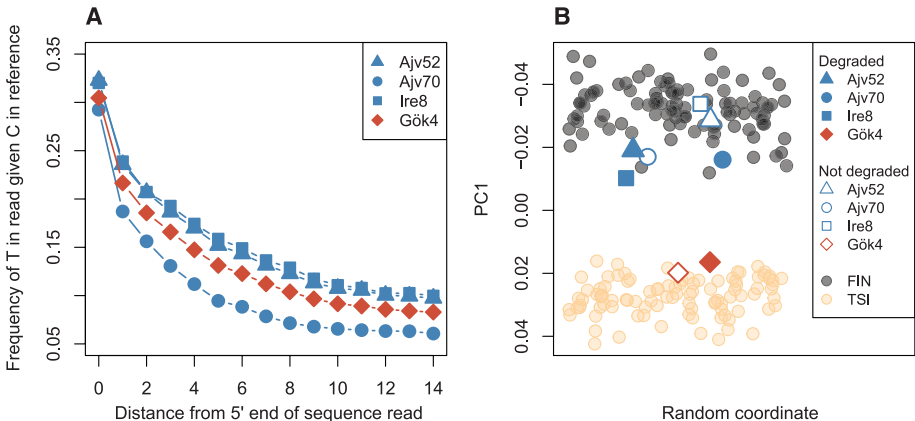


Fig. 2. Assessing authenticity by population genetic analysis of degraded and nondegraded molecules. Authentic ancient DNA molecules show an increased rate of cytosine-to-thymine (C→T) mismatches due to nucleotide misincorporation, at the 5' ends of sequences. We use this feature to test whether sequences with evidence of nucleotide misincorporation and sequences without such evidence have different population genetic affinities, thus providing information on the possible influence of potential modern human contamination. (A) T base frequency in the sequence reads at positions where a C is seen in the human reference genome for all four Neolithic individuals. (B) Procrustes-transformed PCAs of data from each Neolithic individual divided into molecules with a C→T mismatch in the first 10 bp ("degraded": solid red and blue symbols) and molecules without a C→T mismatch in the first 10 bp ("not degraded": open red and blue symbols). TSI, individuals from Italy; FIN, individuals from Finland. The x axis displays random numbers to aid visualization.

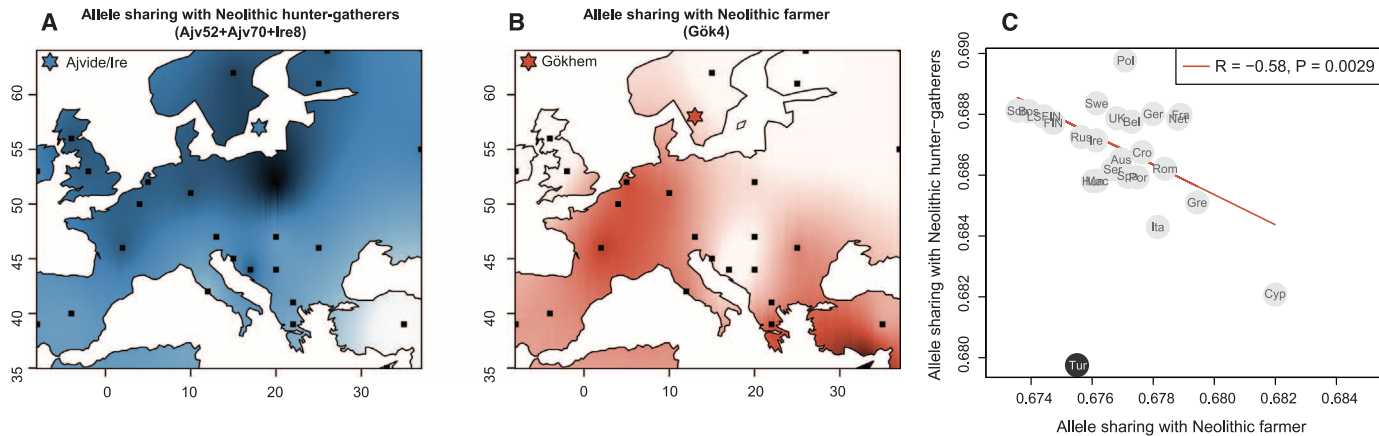


Fig. 3. Allele sharing between Neolithic and extant Europeans. (A) Interpolated allele sharing between the Neolithic hunter-gatherers (pooled, a star shows the sampling location) and extant European populations (black squares). (B) Allele sharing between the Neolithic farmer (Gök4, a star shows the sampling location) and extant European populations (black squares). (C)

In all extant European populations except for Turkey (black disk, excluded from the test of correlation), a high degree of allele sharing with one Neolithic population tends to be associated with a low degree of allele sharing with the other Neolithic population. The population codes are explained in table S10.

the Neolithic hunter-gatherers or the Neolithic farmer (16). The Neolithic hunter-gatherers shared most alleles with northern Europeans, and the lowest allele sharing was with populations from southeastern Europe (Fig. 3A). In contrast, the Neolithic farmer shared the greatest fraction of alleles with southeastern European populations (Cypriots and Greeks) and showed a pattern of decreasing genetic similarity to populations from the northwest and northeast extremes of Europe (Fig. 3B). Individuals from Turkey stand out because of low levels of allele sharing with both Neolithic groups, possibly due to gene flow from outside of Europe, but all other European populations can roughly be represented as a cline in which allele sharing with Neolithic hunter-gatherers is negatively correlated with allele sharing with Neolithic farmers (Fig. 3C).

We also conducted tests of population topology using genealogical concordance (16), and found that Neolithic hunter-gatherers have the strongest affinity to modern Finnish individuals [FIN and “Late Settlement” FIN (LSFIN) (22)], whereas the Neolithic farmer appears most related to extant Mediterranean European populations (fig. S17). However, in formal tests for admixture (31) that assumed the topology (*Out-group*, *Neolithic farmer*), (*X*, *LSFIN*), we found widespread evidence for gene flow between the Neolithic farmer and other European populations (*X*) for various non-European outgroups (table S14). To estimate the extent of this putative gene flow, we constructed a hypothetical model in which each of 14 modern European populations (21, 22, 28) is a mixture of genetic material from a population that is most similar to LSFIN and a second population more related to the Neolithic farmer (Gök4) than to Levantine Druze (fig. S18). We estimated that people of southern, central and northern Swedish descent are, on average, of $41 \pm 8\%$, $36 \pm 7\%$, and $31 \pm 6\%$ Neolithic farmer-related ancestry, respectively (± 1 SE). Across Europe, this fraction decreases from $95 \pm 13\%$ in Sardinians to $52 \pm 8\%$ in the CEU population (individuals of northwestern European descent) and $11 \pm 4\%$ in Russians (table S15). Additionally, on the basis of two complete CEU genomes (25), we estimated similar population divergence times (32) between the CEU population and the Neolithic farmer [9.8 thousand years ago (ka); confidence interval (CI), 1.5 to 18.5 ka] and the Neolithic hunter-gatherers (6.5 ka; CI, 1.5 to 11.5 ka) [(16) fig. S15 and table S11], which is consistent with an intermediate fraction of Neolithic farmer-related ancestry in the CEU population.

In our genomic analyses, the Scandinavian Neolithic hunter-gatherers (PWC) have a genetic profile that is not fully represented by any sampled contemporary population (Fig. 1) and may thus constitute a gene pool that is no longer intact or no longer exists. Although the origin of the Neolithic hunter-gatherers is contentious, the similar mtDNA haplogroup composition of PWC

individuals (8) (Table 1) and Mesolithic and Paleolithic individuals (7, 29) indicates some continuity with earlier European populations, but resolving this hypothesis will require pre-Neolithic genomic data.

A parsimonious model for explaining our results is that farming practices were brought to northern Europe by a group of people that were genetically distinct from resident hunter-gatherers. The alternative explanation—that Gök4 is not typical of the Neolithic farmer population—appears less likely, based on isotopic analyses and burial context (16, 17). Furthermore, the mtDNA haplogroups of Gök4 and other investigated TRB individuals (8) occur among central European Neolithic farmers associated with the LBK culture [*Linearbandkeramik* (9)], a culture with close connection to the TRB culture (5). However, the alternative explanation, that Gök4 is a recent migrant, would still suggest long-range migration across the European continent. Although Neolithic farmers associated with cultures other than TRB could potentially have different histories, the observation that Gök4 is genetically most similar to extant populations found in Mediterranean Europe is in contrast (see e.g. Figs. 1 and 3) to an mtDNA study that suggests extant populations in Turkey and the Near East as being genetically most similar to central European Neolithic farmers (9). The genetic affinity of an individual (Gök4) from the northern frontier of the agricultural expansion to southern Europeans suggests persistent barriers to gene flow between resident and colonizing groups during the initial stages of expansion and settlement, barriers that perhaps became more permeable over time. That gene flow between farmer and hunter-gatherer populations, possibly over a long period, eventually gave rise to the present pattern of genetic variation in Europe is also supported by the observation that most European populations appear genetically intermediate to the two Neolithic groups. Regardless of the underlying model, our study provides direct genomic evidence of stratification between Neolithic cultural groups separated by less than 400 km, differentiation that encapsulates the extremes of modern-day Europe and appears to have been largely intact for ~1000 years after the arrival of agriculture. Thus, the genetic composition of contemporary Europeans may have been shaped by prehistoric migration that drove the expansion of agriculture.

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Supplementary Materials

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Endogenous Protein S-Nitrosylation in *E. coli*: Regulation by OxyR

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Endogenous S-nitrosylation of proteins, a principal mechanism of cellular signaling in eukaryotes, has not been observed in microbes. We report that protein S-nitrosylation is an obligate concomitant of anaerobic respiration on nitrate in *Escherichia coli*. Endogenous S-nitrosylation during anaerobic respiration is controlled by the transcription factor OxyR, previously thought to operate only under aerobic conditions. Deletion of OxyR resulted in large increases in protein S-nitrosylation, and S-nitrosylation of OxyR induced transcription from a regulon that is distinct from the regulon induced by OxyR oxidation. Furthermore, products unique to the anaerobic regulon protected against S-nitrosothiols, and anaerobic growth of *E. coli* lacking OxyR was impaired on nitrate. Thus, OxyR serves as a master regulator of S-nitrosylation, and alternative posttranslational modifications of OxyR control distinct transcriptional responses.

Nitric oxide (NO) influences eukaryotic cellular processes in large part through protein S-nitrosylation, and aberrant S-nitrosylation generates a nitrosative stress that is a component of multiple diseases (1–3). Although microorganisms may be exposed to NO, which is both a component of host defense mechanisms (4) and a minor byproduct of anaerobic metabolism (5), endogenous S-nitrosylation has not been reported. We demonstrate that S-nitrosylation is a prominent feature of anaerobic respiration in *Escherichia coli* and describe a regulon that regulates endogenous S-nitrosylation.

Wild-type (WT) *E. coli* were grown anaerobically in minimal media containing either 10 mM fumarate or nitrate, and S-nitrosylation was analyzed in lysates by using photolysis-chemiluminescence (6), which measures NO displaced selectively from Cys residues, and the biotin-switch method (6), in which biotinylation identifies S-nitrosylated cysteines (S-nitrosothiols, SNOs). Whereas SNOs were not detectable in fumarate-grown cells, cells respiring anaerobically in the presence of nitrate (ARN) accumulated S-nitrosylated proteins (SNO-proteins) (Fig. 1, A and B). When *E. coli* were grown on fumarate and then switched to medium containing nitrate (10 mM), SNOs accumulated progressively (fig. S1). Bacteria respiring anaerobically on nitrate can catalyze nitrosation of amino dyes when supplemented with high concentrations of exogenous nitrite (5, 7), but in our experiments, endogenous proteins underwent S-nitrosylation without exogenous nitrite.

Although S-nitrosylation chemistry is classically O₂-dependent (8), anaerobic S-nitrosylation after addition of NO to eukaryotic cells has been

shown, which involves the participation of transition metals (9, 10). Anaerobic exposure of bacteria to the NO donor diethylamine NONOate (DEANO) (100 μ M, 30 min) resulted in the accumulation of SNOs (8.9 $\pm$ 2.0 nmol SNO per mg protein), and prior treatment of cells with a chelator of divalent metal cations, 1, 10-phenanthroline (Phen) (1 mM, 30 min) decreased the formation of SNOs and of other photolabile nitrosylated species (XNOs, known to include transition metal-NO species) (fig. S2A). Pretreatment of bacteria with Phen also decreased formation of SNOs during ARN (fig. S2A). NrfA (nitrite reductase) and NarG (nitrate reductase) are potential sources of NO (5, 7, 11) implicated in N-nitrosation of exogenous probes (5, 12). Amounts of SNO were slightly lower in Δ nrfA versus WT *E. coli* but were reduced by about 80% in the Δ narG strain (fig. S2B); nitrite levels were somewhat reduced but remained at about 4 mM in Δ narG cells (fig. S2C). Thus, the NarG nitrate reductase appears to be a major source of S-nitrosylating activity in *E. coli* under anaerobic conditions, and S-nitrosylation is largely independent of nitrite concentration per se and of nitrite reduction by NrfA.

Amounts of endogenous SNO-proteins observed in WT cells during ARN were comparable to those in WT cells after exposure to 200 μ M S-nitrosocysteine (CysNO) (Fig. 1C), which results in protein S-nitrosylation sufficient to generate a growth-impairing nitrosative stress (13). Amounts of SNO-proteins were also higher in Δ oxyR cells than in WT cells during ARN (Fig. 1C). The Δ oxyR cells grew more slowly than WT cells on nitrate, and this growth defect was rescued by introduction of a plasmid overexpressing OxyR (whereas OxyR rescue had no effect on growth on fumarate) (Fig. 1D). Thus, the well-known growth-promoting effect of nitrate vis-à-vis fumarate (14) is dependent on OxyR. The Δ oxyR strain was also significantly more sensitive to growth inhibition by exogenous S-nitrosoglutathione (GSNO) (Fig. 1E and fig. S3). Amounts of SNO-protein were higher in Δ oxyR cells than in WT cells after exposure to exog-

enous CysNO, which reflected reduced denitrosylation (SNO clearance) (Fig. 1C). Thus, protein S-nitrosylation during ARN generates a nitrosative stress that is ameliorated by OxyR, and OxyR (and possibly other genes in its regulon) appears to protect against nitrosative stress at least in part by augmenting protein denitrosylation.

Under aerobic conditions, OxyR can be activated in vitro by oxidation or nitrosylation of a critical regulatory Cys thiol (15), but S-oxidation (which is mediated by hydrogen peroxide) would be inconsistent with anaerobiosis. To test for endogenous S-nitrosylation of OxyR, we expressed FLAG-tagged OxyR in Δ oxyR cells and assayed SNO-OxyR-FLAG with the SNO-RAC (resin-assisted capture) method (6), which captures SNO-proteins on resin. SNO-OxyR-FLAG was detected in cells growing anaerobically on nitrate but not on fumarate (Fig. 1F). The extent of OxyR S-nitrosylation during ARN was comparable to that resulting from exposure to 200 μ M CysNO (Fig. 1G and fig. S4, A and B), and this S-nitrosylation by CysNO was also rapidly reversed by endogenous denitrosylation (Fig. 1G). Treatment with chloramphenicol to block translation before addition of CysNO did not affect total amounts of OxyR (fig. S4C), which ruled out preferential degradation of SNO-OxyR. We found no apparent differences in rates of oxyR transcription between WT cells grown anaerobically on nitrate or fumarate (fig. S5). Thus, nitrosative stress that is a concomitant of ARN is sensed by OxyR S-nitrosylation.

To identify OxyR-dependent genes that showed increased transcription under conditions that induce S-nitrosylation, we performed a global transcriptome analysis of WT versus Δ oxyR *E. coli* grown anaerobically on either nitrate or fumarate (10 mM). Stringency cutoffs were set to identify genes in WT *E. coli* that were induced at least twofold on nitrate versus fumarate (tables S1 and S2). The 145 genes identified were clustered by using dChip microarray analysis software (16) (Fig. 2A). A majority (129 genes) were OxyR-dependent—that is, they did not show increased transcription during ARN in Δ oxyR cells (Fig. 2, A and B; fig. S6A; and tables S1 and S2) or, in a small subset, were more highly expressed on fumarate in Δ oxyR versus WT (fig. S6B). Thus, the phenotype that distinguishes anaerobic growth on nitrate from anaerobic growth on fumarate is in large part OxyR-dependent. The remaining genes showed increased expression in both WT and Δ oxyR (Fig. 2, A and B, and tables S1 and S2) or only in Δ oxyR (table S3). The 20 genes with the greatest increase in transcription during ARN in WT *E. coli* were further validated by quantitative real-time polymerase chain reaction (QPCR) (Fig. 2B); they include many activated in response to nitrosative stress, including *hmp*, *hcp-hcr*, *yeaR-yoaG*, and *ytfE* (17–21). The genes with greatest transcriptional change in the Δ oxyR strain (table S3) are directly responsive to NO concentration (22, 23), including those encoding the NO-metabolizing enzymes *hmp* (24) and *norV/norW* (25); further, enhanced transcription of *norV/norW*

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was observed only in $\Delta oxyR$. Thus, WT *E. coli* exhibit a genetic signature of nitrosative stress during ARN, and an additional set of protective genes are highly expressed in the absence of OxyR.

We used *E. coli* Entry Point (http://coli.berkeley.edu/cgi-bin/ecoli/coli_entry.pl) to group the ~150 OxyR-dependent genes identified by transcriptome analysis into known or predicted operons. The transcription of ~60 genes or operons was dependent on OxyR under anaerobic conditions (tables S1 and S2). OxyR-dependent genes identified in this analysis may include genes that respond directly to OxyR, as well as genes or operons that may be activated by other OxyR-dependent

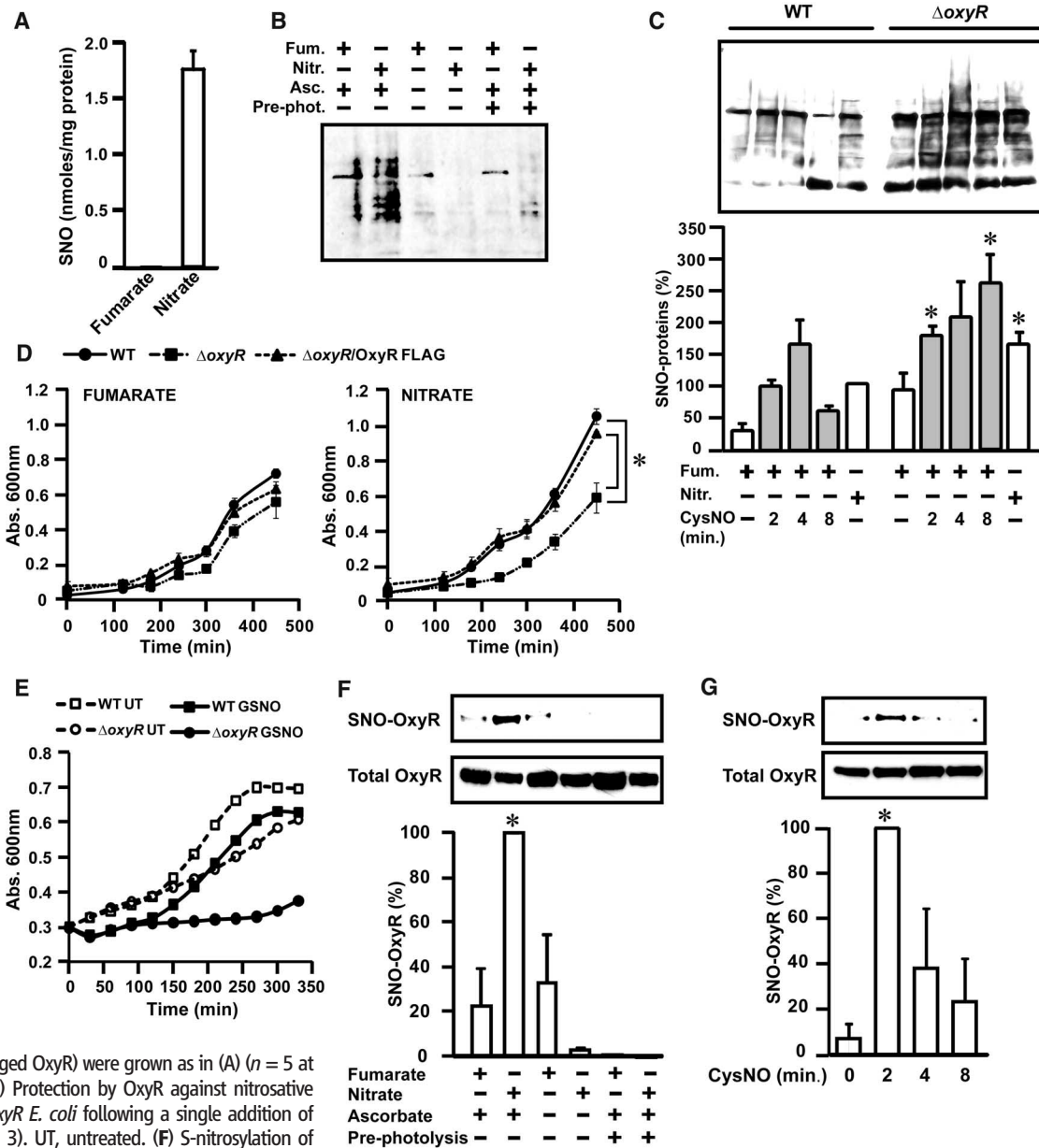
genes (and does not preclude codependence on other transcriptional regulators). Most of the genes or operons had not been previously identified as being OxyR-dependent. Thus, OxyR controls the expression of a large set of genes that form a nitrosative stress regulon that is distinct from the OxyR regulon activated by oxidative stress.

To elucidate the mechanism of OxyR activation during ARN, we focused on *hcp* (encoding a hybrid cluster protein), which is highly induced in an OxyR-dependent manner (40-fold versus 5-fold in WT versus $\Delta oxyR$) (table S1). In WT cells, transcription of *hcp* was increased under both aerobic and anaerobic conditions by treatment with

CysNO, GSNO, or DEANO but not by hydrogen peroxide (H_2O_2), diamide, or oxidized glutathione (Fig. 3A and fig. S7A). Transcription was not increased in $\Delta oxyR$ cells (Fig. 3A). By contrast, genes in the known oxidative stress regulon of OxyR (*katG*, *sufA*, and *grxA*) (26) showed increased transcription in response to oxidants (fig. S7B). These results indicate that *hcp* responded to OxyR activated by S-nitrosylation rather than S-oxidation (S-OH, S-S, S-SG). Modification-specific transcriptional activation by OxyR of the *hcp* promoter was confirmed by in vitro transcription assay with purified redox forms of OxyR (Fig. 3B). Reduced and S-oxidized OxyR had,

Fig. 1. Accumulation of

SNO-proteins and nitrosative stress in *E. coli* respiring anaerobically on nitrate. (A) Endogenous S-nitrosylation in *E. coli* growing anaerobically on nitrate. *E. coli* were grown in minimal medium containing either fumarate or nitrate (10 mM) and harvested during mid-log phase (absorbance at 600 nm of 0.4). The SNO content of lysates was determined by mercury-coupled photolysis-chemiluminescence ($n = 3$, $\pm$ SEM). (B) SNO-proteins generated during anaerobic growth. *E. coli* were grown as in (A), and lysates were subjected to biotin-switch and Western blotting with NeutrAvidin-horseradish peroxidase. Ascorbate ($\pm$) and pre-ultraviolet photolysis (pre-phot.) controls were included. (C) Regulation by OxyR of protein S-nitrosylation and denitrosylation. WT and $\Delta oxyR$ *E. coli* were grown anaerobically as in (A). As indicated, cells growing on fumarate were treated with CysNO (200 μ M), and SNO-proteins in lysates were visualized by biotin-switch as in (B). Results represent three to five separate experiments ($\pm$ SEM). * $P < 0.05$ WT versus $\Delta oxyR$. (D) Impaired growth of OxyR-null bacteria on nitrate. WT, $\Delta oxyR$ and $\Delta oxyR$ /OxyR-FLAG ($\Delta oxyR$ overexpressing FLAG-tagged OxyR) were grown as in (A) ($n = 5$ at each time point; * $P < 0.05$). (E) Protection by OxyR against nitrosative stress. Growth of WT and of $\Delta oxyR$ *E. coli* following a single addition of GSNO (125 μ M) at time 0 ($n = 3$). UT, untreated. (F) S-nitrosylation of OxyR during anaerobic growth on nitrate. $\Delta oxyR$ /OxyR-FLAG *E. coli* were grown as in (A). SNO-OxyR in lysates was analyzed by SNO-RAC ($n = 4$, $\pm$ SEM). *Differs from fumarate control by analysis of variance (ANOVA) with Dunnett's test, $P < 0.05$. Control samples were generated by omission of ascorbate or by pre-photolysis. (G) Dynamic S-nitrosylation and denitrosylation of OxyR. $\Delta oxyR$ /OxyR-FLAG *E. coli* grown anaerobically on 10 mM fumarate as in (A) were treated with a single addition of CysNO (200 μ M). Lysates were analyzed by SNO-RAC ($n = 4$, $\pm$ SEM). *Differs from 0 min control by ANOVA with Dunnett's test, $P < 0.05$.



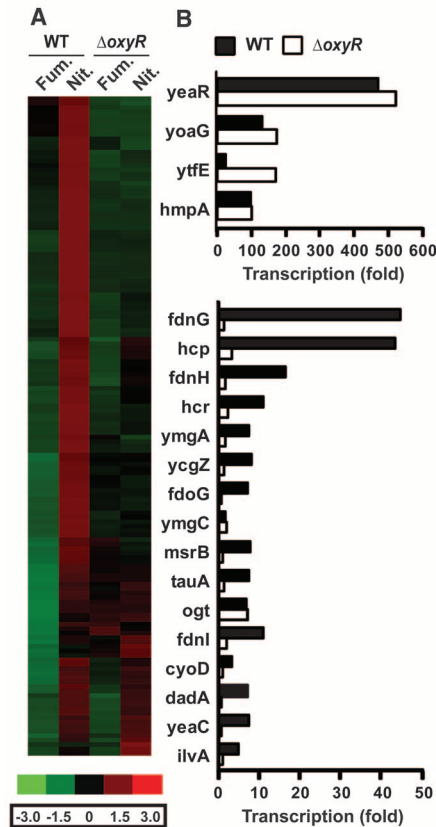


Fig. 2. Microarray analysis of gene expression in *E. coli* growing anaerobically. (A) Expression of OxyR-regulated genes on nitrate versus fumarate. Genes in WT cells induced >2.0-fold on nitrate (left panel); a large majority are OxyR-dependent. GenspringGX software (Agilent) was used to analyze microarray results, and genes were clustered by using dChip. (B) Validation by QPCR of the top 20 genes regulated anaerobically on nitrate in WT *E. coli* and the role of OxyR. Cells were grown anaerobically on nitrate or fumarate before QPCR. Fold expression of each gene on nitrate was calculated relative to expression on fumarate by using the comparative C_t method. Expression in each sample was normalized to that of 16S rRNA ($n = 2$).

respectively, one and no free thiol per molecule by dithionitrobenzoic acid assay. SNO-OxyR had one SNO per molecule as shown by photolysis-chemiluminescence that was localized by mass spectrometry to Cys¹⁹⁹, and all other thiols were in the reduced form (figs. S8 to S11, supplementary text).

Binding of OxyR to the *hcp* promoter was verified by electrophoretic mobility shift assay, and the interaction was inhibited by DNA containing the *katG* OxyR binding site (fig. S12). SNO-OxyR strongly induced transcription at the *hcp* promoter (Fig. 3B). Transcriptional activity was decreased both by dithiothreitol (DTT) (100 mM) and by ascorbate, which denitrosylate proteins (Fig. 3B). S-oxidized OxyR did not stimulate transcription at the *hcp* promoter (Fig. 3B), whereas both S-oxidized and S-nitrosylated

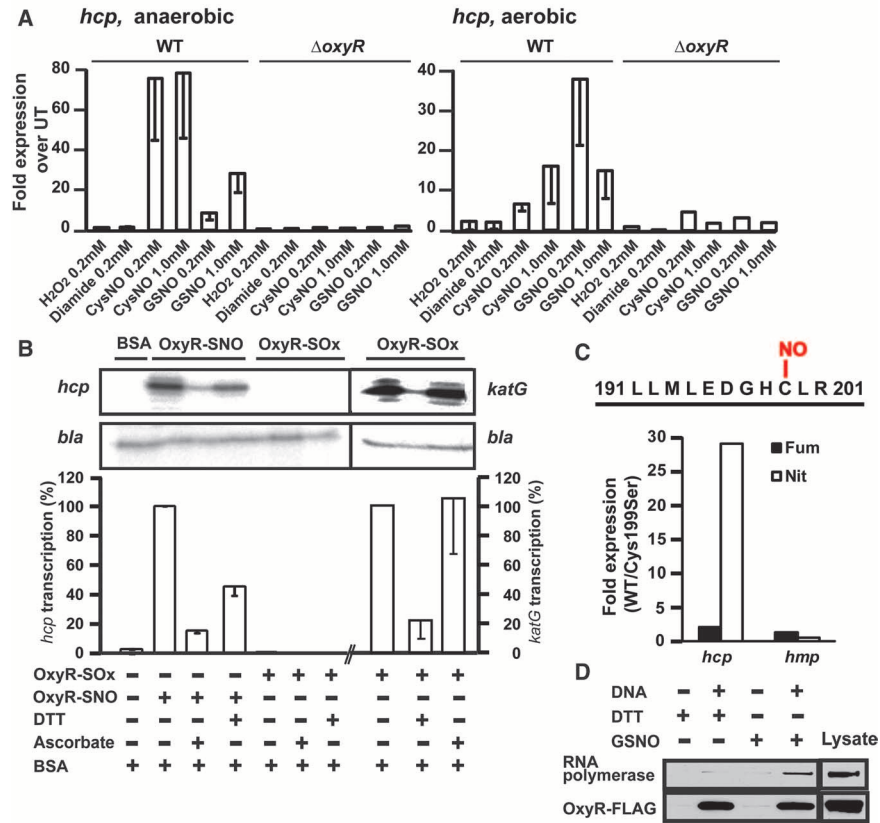


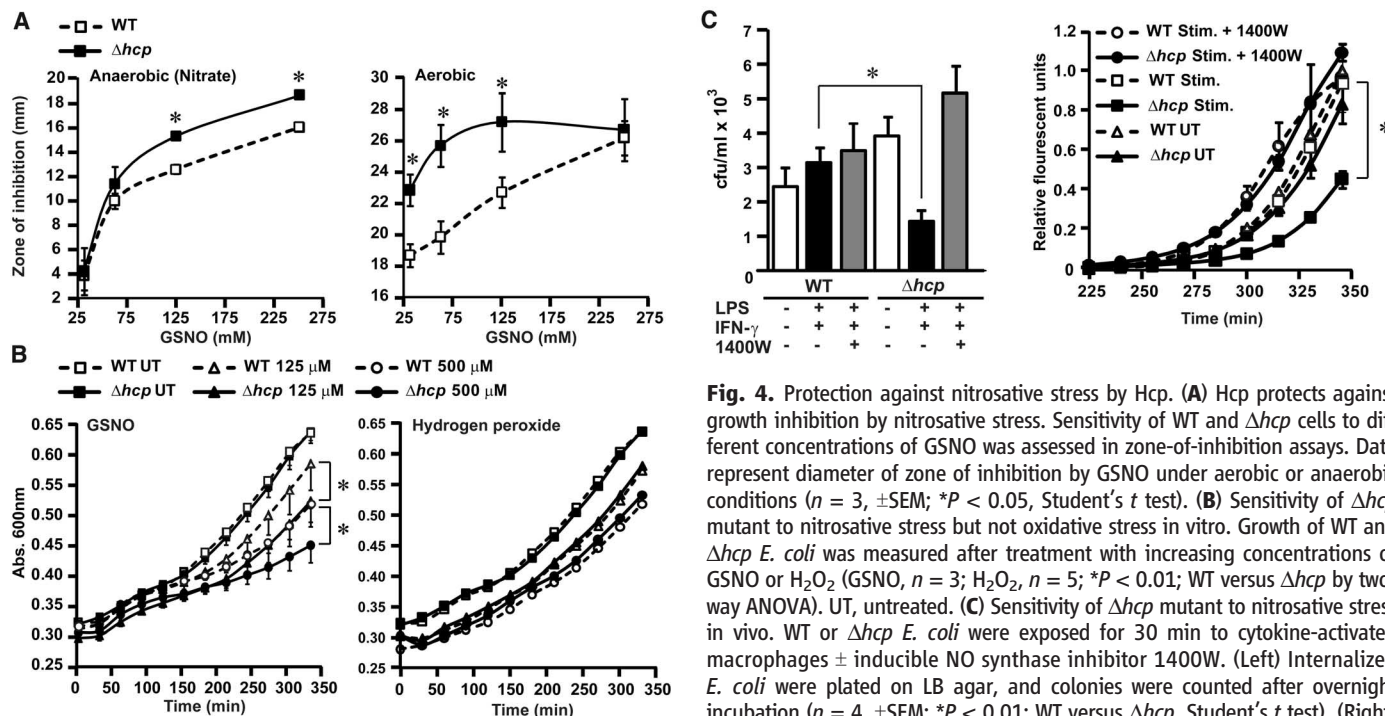
Fig. 3. Activation of *hcp* transcription by SNO-OxyR. (A) Increased *hcp* gene expression induced by SNOs. *E. coli* (WT and $\Delta oxyR$) were grown aerobically or anaerobically to mid-log phase and treated as indicated for 5 min. Expression levels of *hcp* RNA were determined by QPCR (WT $n = 3$, $\pm$ SEM; $\Delta oxyR$ $n = 2$). UT, untreated. (B) In vitro transcription at *hcp* promoter by S-nitrosylated but not S-oxidized OxyR. Primer extension products of in vitro transcription reactions with S-nitrosylated and S-oxidized OxyR are shown. A plasmid containing the *hcp* gene [and the β -lactamase (*bla*) gene] was used as the template. DTT and ascorbate were used at 100 mM. Results are expressed as percent induction by SNO-OxyR, normalized with respect to *bla* control ($n = 3$ separate SNO-OxyR preparations, $\pm$ SEM). Activation of *katG* transcription by the same preparation of OxyR-SOx is also shown (grouping of images from within the same gel is indicated by vertical dividing lines). BSA, bovine serum albumin. (C) Dependence of *hcp* expression during ARN on OxyR Cys¹⁹⁹. Cys¹⁹⁹ was identified as the sole site of S-nitrosylation in OxyR by mass spectrometry (see figs. S9 and S10, supplementary text). $\Delta oxyR$ /OxyR-FLAG and $\Delta oxyR$ /C199S-OxyR-FLAG *E. coli* were grown anaerobically on nitrate or fumarate. Fold expression of *hcp* (OxyR-dependent) and of *hmp* (OxyR-independent) on fumarate or nitrate in OxyR-FLAG (WT) versus C199S-OxyR-FLAG cells was calculated by using the comparative C_t method. Expression levels were normalized to 16S rRNA ($n = 2$ experiments). (D) Interaction of SNO-OxyR with *hcp* promoter and recruitment of RNA polymerase. $\Delta oxyR$ /OxyR-FLAG lysate was incubated with a biotin-labeled *hcp* promoter fragment in the presence of DTT or GSNO. Proteins bound to DNA were pulled down by streptavidin beads and visualized by Western blotting with antibodies to FLAG and to RNA polymerase β' . The results are representative of three experiments.

OxyR activated the *katG* promoter (Fig. 3B and fig. S13). An essential role for S-nitrosylation of OxyR Cys¹⁹⁹ in *hcp* induction during ARN was demonstrated by the finding that transcription of *hcp* was not increased during ARN in $\Delta oxyR$ *E. coli* overexpressing a mutant form of OxyR in which Ser replaces Cys at position 199 (Cys199Ser) (Fig. 3C). Collectively, these data establish that *hcp* transcription is selectively activated by S-nitrosylation of OxyR in vitro and during ARN.

Oxidized OxyR activates transcription by recruiting RNA polymerase to promoters (27). We used a biotin-labeled *hcp* promoter DNA frag-

ment and evaluated its interaction with SNO-OxyR and RNA polymerase (Fig. 3D). OxyR treated with DTT or GSNO (to generate reduced and S-nitrosylated OxyR, respectively) bound to promoter DNA (Fig. 3D), but only S-nitrosylated OxyR recruited RNA polymerase, consistent with transcriptional activation by the S-nitrosylated but not reduced OxyR (15).

At least some OxyR-dependent genes induced during ARN serve to protect against endogenous nitrosative stress (Fig. 1D). However, the anaerobic OxyR regulon (Fig. 2) does not include genes known to ameliorate nitrosative stress (2). To evaluate the potential protective function of *hcp* as



assay ($n = 3$, $\pm$ SEM; * P < 0.01; WT versus Δhcp , two-way ANOVA). Increased sensitivity to macrophage-derived nitrosative stress in Δhcp cells was not evident after prolonged incubations (>1 hour; not shown). IFN- γ , interferon- γ .

an exemplar, we assessed the survival of Δhcp and WT cells on agar plates in the presence of GSNO placed in cups in the agar. Under both aerobic and anaerobic conditions, the Δhcp strain had significantly (P < 0.05) larger zones of inhibition compared with those of the WT cells (Fig. 4A). Δhcp cells were also significantly more sensitive to GSNO in growth assays (Fig. 4B). By contrast, there was no statistically significant difference in the growth rates of WT and Δhcp cells treated with H₂O₂ (Fig. 4B). Growth inhibition by exposure to GSNO was comparable in Δhcp *E. coli* and *E. coli* lacking Hmp, a major antinitrosative enzyme (fig. S14). In addition, growth of Δhcp cells was impaired in the presence of activated macrophages, and growth differences between WT and Δhcp cells were eliminated by blocking macrophage NO production (Fig. 4C). Thus, Hcp appears to protect selectively against nitrosative stress.

We found that endogenous protein S-nitrosylation is a concomitant of ARN, a major mode of existence in diverse microorganisms. The functioning of S-nitrosylation independent of NO synthases or O₂ indicates that it may be a conserved mechanism of primordial origin for control of protein function. S-nitrosylation appears to convey an endogenous nitrosative stress during anaerobic metabolism, analogous to the oxidative stress entailed by oxidative (aerobic) metabolism. The transcription factor OxyR, activated by oxidation under aerobic conditions, is also activated by S-nitrosylation under anaerobic conditions and thereby induces expression in a distinct regulon,

which reveals that a transcriptional activator can independently control two distinct regulons through alternative posttranslational modifications. Components of the regulon induced by S-nitrosylated OxyR (including *hcp*) protect against endogenous nitrosative stress, and protection is conferred at least in part by limiting S-nitrosylation. Thus, OxyR regulates S-nitrosylation in *E. coli*. The ability of proteins to respond differentially to alternative modifications of Cys thiol may represent the basis of a molecular code for redox regulation.

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Supplementary Materials

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Tables S1 to S4
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Function and Molecular Mechanism of Acetylation in Autophagy Regulation

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Protein acetylation emerged as a key regulatory mechanism for many cellular processes. We used genetic analysis of *Saccharomyces cerevisiae* to identify Esa1 as a histone acetyltransferase required for autophagy. We further identified the autophagy signaling component Atg3 as a substrate for Esa1. Specifically, acetylation of K19 and K48 of Atg3 regulated autophagy by controlling Atg3 and Atg8 interaction and lipidation of Atg8. Starvation induced transient K19-K48 acetylation through spatial and temporal regulation of the localization of acetylase Esa1 and the deacetylase Rpd3 on pre-autophagosomal structures (PASs) and their interaction with Atg3. Attenuation of K19-K48 acetylation was associated with attenuation of autophagy. Increased K19-K48 acetylation after deletion of the deacetylase Rpd3 caused increased autophagy. Thus, protein acetylation contributes to control of autophagy.

Autophagy is a degradation pathway conserved from yeast to mammals (1). Eighteen essential autophagy genes have been identified in yeast (2). Acetylation is reported to regulate autophagy (3, 4). Treating cells with deacetylase inhibitors can induce autophagy (5), whereas P300-mediated acetylation of autophagy proteins appears to have an inhibitory role in autophagy (3). Thus, the role of acetylation in autophagy remains unclear.

We investigated whether any of the eight histone acetyltransferase (HAT) complexes in *Saccharomyces cerevisiae* are required for autophagy. By analyzing deletion or mutants of catalytic subunits of all eight HATs, we identified Esa1, the catalytic subunit of NuA4, as a HAT that is required for autophagy (Fig. 1A). Nitrogen starvation-induced green fluorescent protein (GFP)–Atg8 vacuoles translocation and cleavage were impaired in *esa1-1*, a temperature-sensitive mutant strain of

Esa1 (Fig. 1, B to D), which indicated that Esa1 regulates autophagy. Epl1, a regulatory subunit of NuA4, is also required for autophagy (Fig. 1, E to G). Autophagy induced by other cues was also impaired in *esa1-1* or *epl1-1* (figs. S1 and S2), and depletion of Tip60, the mammalian homolog of Esa1, impaired starvation-induced autophagy in normal rat kidney (NRK) cells (fig. S3). Thus, Esa1-mediated acetylation is required in an evolutionarily conserved manner for autophagy.

We tested whether Esa1 regulates autophagy by directly acetylating autophagy proteins. The 18 essential autophagy genes can be classified into several groups according to their function (2). By testing which function is impaired in *esa1-1*, we could narrow our search for the Esa1 substrate (fig. S4A). Rapamycin-induced autophagy was impaired in *esa1-1* (fig. S4, B and C), indicating

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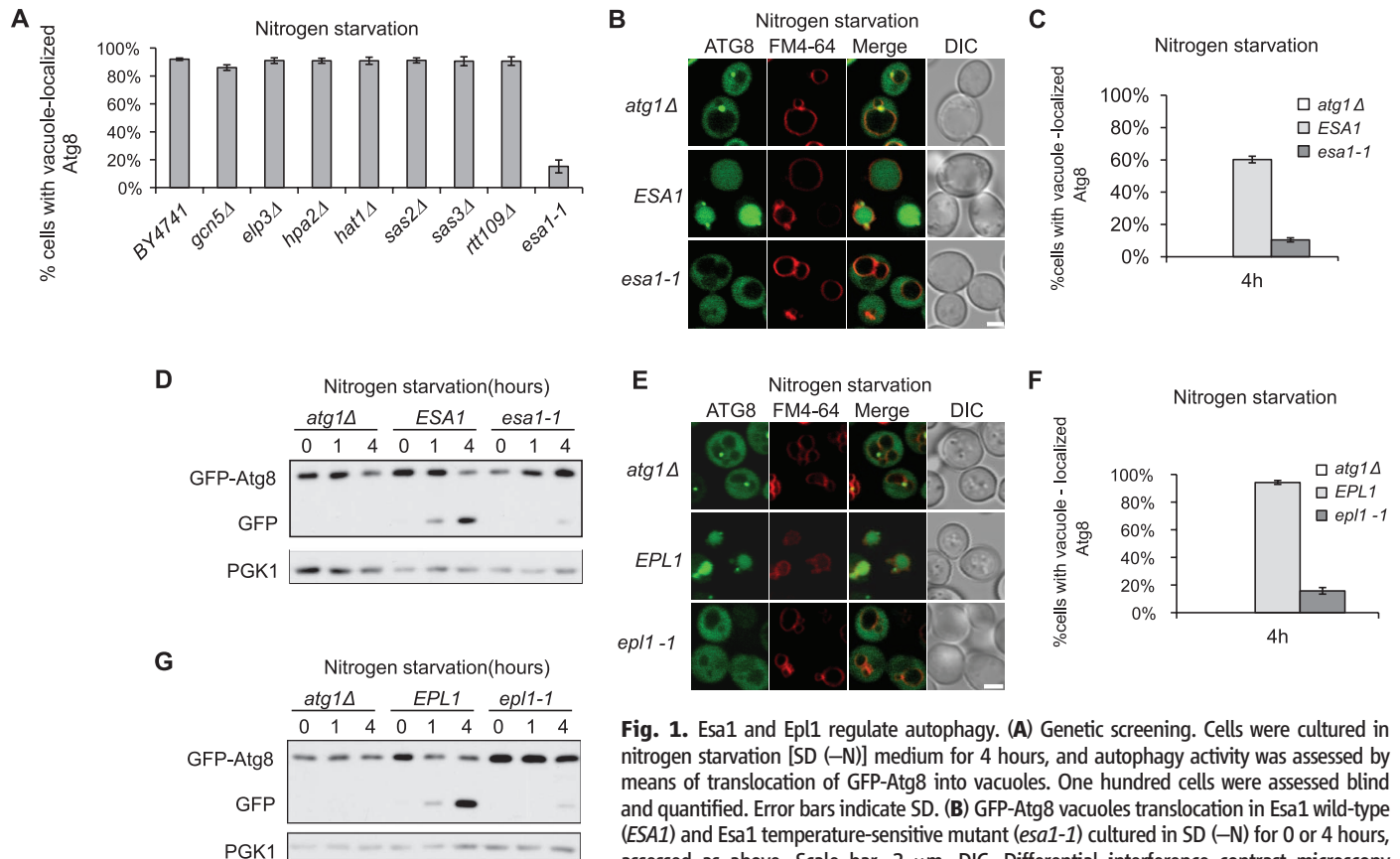


Fig. 1. Esa1 and Epl1 regulate autophagy. **(A)** Genetic screening. Cells were cultured in nitrogen starvation [SD (–N)] medium for 4 hours, and autophagy activity was assessed by means of translocation of GFP-Atg8 into vacuoles. One hundred cells were assessed blind and quantified. Error bars indicate SD. **(B)** GFP-Atg8 vacuoles translocation in *Esa1* wild-type (*ESA1*) and *Esa1* temperature-sensitive mutant (*esa1-1*) cultured in SD (–N) for 0 or 4 hours, assessed as above. Scale bar, 2 μm. DIC, Differential interference contrast microscopy images. **(C)** One hundred cells from **(B)** were quantified for autophagy as above. **(D)** Cells from **(B)** were analyzed by means of Western blot for GFP-Atg8 cleavage. **(E)** GFP-Atg8 vacuoles translocation in *EPL1* and *epl1-1* cells was assessed as above. Scale bar, 2 μm. **(F)** One hundred cells from **(E)** were quantified for autophagy as above. **(G)** Cells from **(E)** were analyzed by means of Western blot for GFP-Atg8 cleavage.

that acetylation regulates molecular events downstream of the mammalian target of rapamycin (mTOR) complex. Atg1 protein puncta formation was normal in *esa1-1* (fig. S4, D and E); formation of Atg8 puncta was reduced in *esa1-1* but not in cells expressing a catalytically inactive form of the kinase Atg1 (fig. S4F), suggesting that acetylation probably regulates steps downstream of the Atg1 complex. Normal phosphatidylinositol 3-phosphate (PI3P) production and Atg6 localization in *esa1-1* indicated that the function of the PI3K complex was unchanged in *esa1-1* (fig. S4, G and H). Thus, the two ubiquitin-like pathways probably contain the substrate of Esa1.

We directly tested the acetylation status of proteins from the two ubiquitin-like pathways; Atg3, Atg5, and Atg8 are acetylated when grown in nutrient-rich medium (Fig. 2A). After starva-

tion, acetylation of Atg3 increased, although the acetylation of other proteins was reduced or unchanged (Fig. 2A). Acetylation level of Atg3 is reduced in *esa1-1* (Fig. 2B), indicating that Atg3 may be a substrate of Esa1. To determine the acetylation site of Atg3, we purified a fusion protein in which Atg3 was fused to glutathione S-transferase (GST-Atg3) from starved yeast (fig. S5); mass spectrometry showed that K19, K48, and K183 were acetylated (Fig. 2C). (Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. In the mutants, other amino acids were substituted at certain locations; for example, K19R indicates that lysine at position 19 was replaced by arginine.) We

also analyzed mutants expressing Atg3 proteins in which acetylated lysines were replaced by arginines. Acetylation of Atg3^{K19R-K48R} and Atg3^{K19R-K48R-K183R} mutants was reduced (Fig. 3A). In vitro Esa1 acetylates Atg3 and acetylation of Atg3^{K19R-K48R} and Atg3^{K19R-K48R-K183R} was reduced (Fig. 3B).

To test which acetylation site is critical for autophagy, we expressed plasmids encoding ATG3 [wild-type (WT)], *atg3*^{K19R}, *atg3*^{K48R}, *atg3*^{K183R}, *atg3*^{K19R-K48R}, and *atg3*^{K19R-K48R-K183R} in cells in which the Atg3 gene was deleted. Autophagy in *atg3Δ* cells was fully rescued by expression of wild-type Atg3. However, cells expression of *atg3*^{K19R-K48R} or *atg3*^{K19R-K48R-K183R} had impaired autophagy induced by various cues (Fig. 3, C to E, and fig. S6), suggesting that K19-K48 acetylation of Atg3 is critical for autophagy.

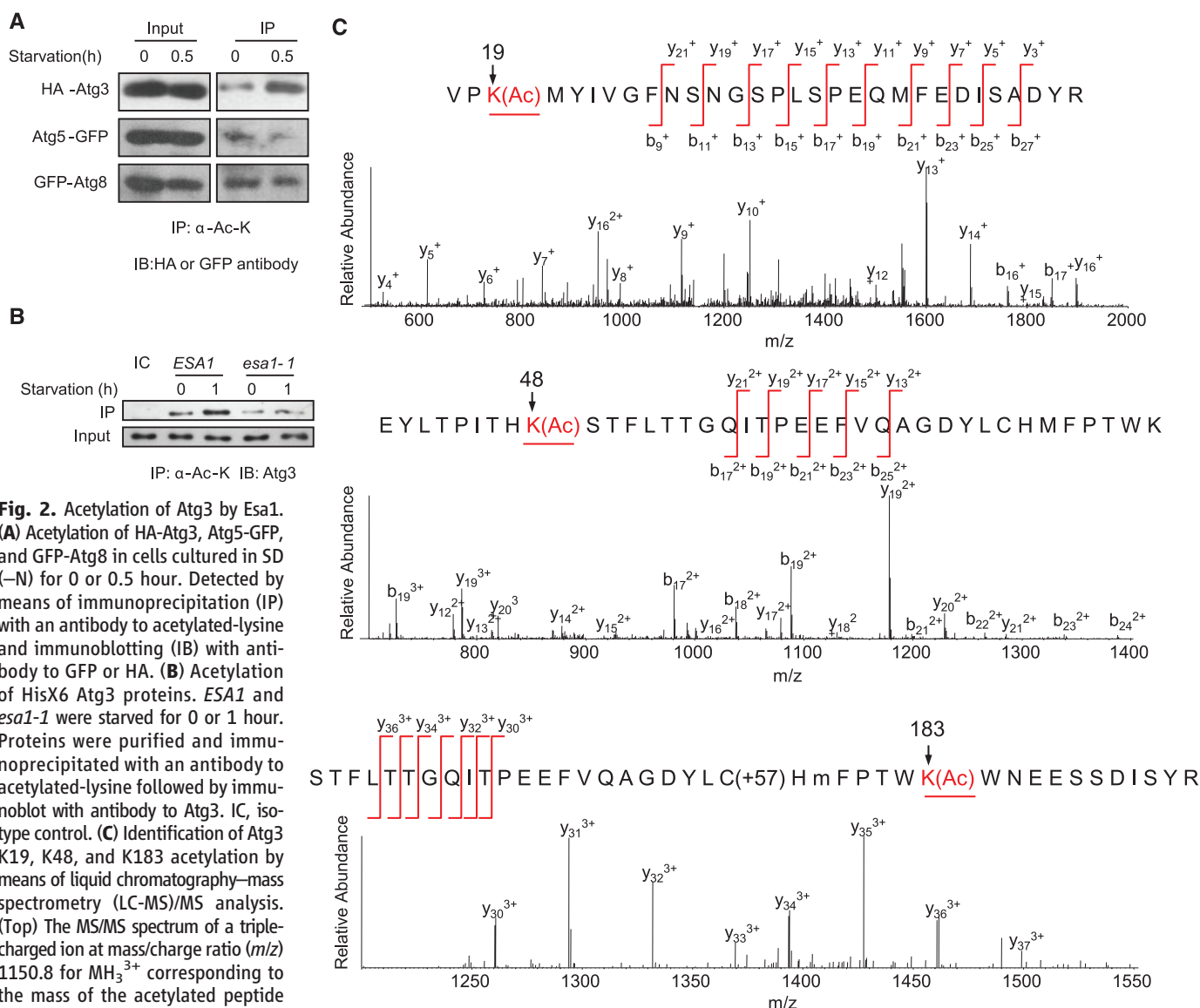


Fig. 2. Acetylation of Atg3 by Esa1. (A) Acetylation of HA-Atg3, Atg5-GFP, and GFP-Atg8 in cells cultured in SD (–N) for 0 or 0.5 hour. Detected by means of immunoprecipitation (IP) with an antibody to acetylated-lysine and immunoblotting (IB) with antibody to GFP or HA. (B) Acetylation of HisX6 Atg3 proteins. *ESA1* and *esa1-1* were starved for 0 or 1 hour. Proteins were purified and immunoprecipitated with an antibody to acetylated-lysine followed by immunoblot with antibody to Atg3. IC, isotype control. (C) Identification of Atg3 K19, K48, and K183 acetylation by means of liquid chromatography–mass spectrometry (LC-MS)/MS analysis. (Top) The MS/MS spectrum of a triple-charged ion at mass/charge ratio (m/z) 1150.8 for MH_3^{3+} corresponding to the mass of the acetylated peptide VPK(Ac)MY–ADYR. The labeled peaks correspond to masses of y ions of acetylated peptide fragments. (Middle) The MS/MS spectrum of a quintuple-charged ion at m/z 907.1 for MH_5^{5+} corresponding to the mass of the acetylated peptide EYLTPITHK(Ac)–TWK.

(Bottom) The MS/MS spectrum of a quadruple-charged ion at m/z 1208.05 for MH_4^{4+} corresponding to the mass of the acetylated peptide STFL–WK(Ac)WNEESSDISYR.

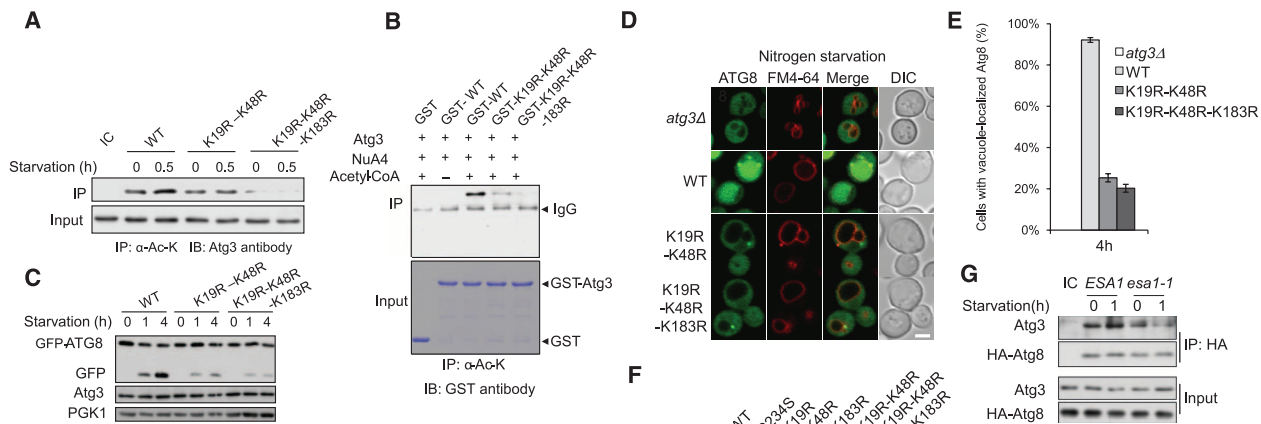


Fig. 3. Effect of Atg3 acetylation on Atg3-Atg8 interaction. **(A)** Acetylation of Atg3 mutants. *atg3Δ+ATG3* WT (WT), *atg3Δ+atg3^{K19R-K48R}* (K19R-K48R), and *atg3Δ+atg3^{K19R-K48R-K183R}* (K19R-K48R-K183R) were starved for 0.5 hour. Proteins from lysates were immunoprecipitated with antibody to acetylated-lysine followed by immunoblotting with antibody to Atg3. **(B)** In vitro acetylation of purified GST, GST-Atg3 WT, GST-Atg3^{K19R-K48R}, and GST-Atg3^{K19R-K48R-K183R} proteins was measured by means of immunoprecipitation with antibody to acetylated-lysine followed by immunoblotting with GST antibody. **(C and D)** Autophagy in *atg3Δ*, *atg3Δ+ATG3* WT, *atg3Δ+atg3^{K19R-K48R}*, and *atg3Δ+atg3^{K19R-K48R-K183R}* cells cultured in SD (–N) for various times were analyzed by means of Western blot for (C) GFP-Atg8 cleavage and (D) translocation of GFP-Atg8 into vacuoles. Scale bar, 2 μm. **(E)** One hundred cells from (D) were quantified for autophagy. **(F)** Lipidation

of Atg8. Purified Atg3 and Atg3 mutants, Atg7, Atg8, and liposomes were incubated for in vitro Atg8 lipidation and analyzed by SDS–polyacrylamide gel electrophoresis. **(G and H)** Association of Atg3 and Atg8 in (G) *ESA1* and *esa1-1* or (H) *atg3Δ+ATG3* WT and *atg3Δ+atg3^{K19R-K48R}* cells expressing HA-Atg8. Cells were cultured in SD (–N) for 0 or 1 hour. Cell lysates were immunoprecipitated with antibody to HA and analyzed by means of Western blot by using an antibody to Atg3.

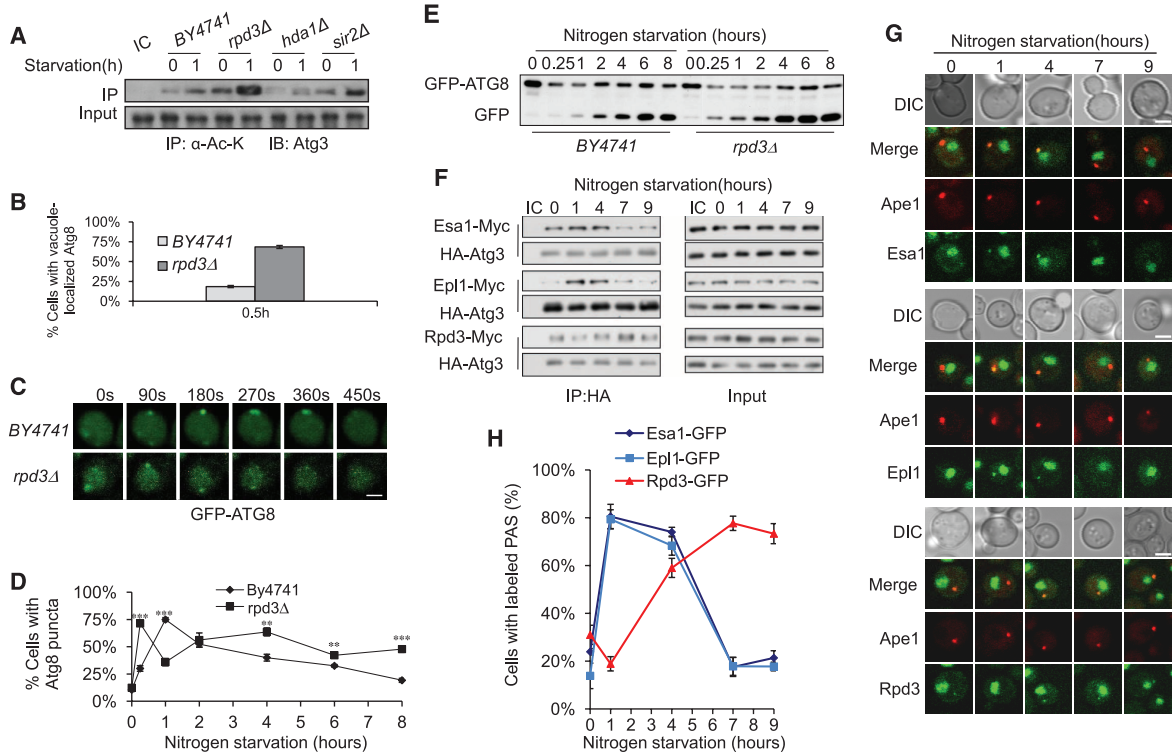


Fig. 4. Effect of acetylation on duration of starvation-induced autophagy, and dynamic Atg3 interaction and PAS localization of Esa1, Epl1, and Rpd3. **(A)** Acetylation of GST-Atg3 in wild type, *rpd3Δ*, *hda1Δ*, and *sir2Δ* expressing GST-Atg3. Cells were starved for 0 or 1 hour. Total GST-Atg3 was purified and immunoprecipitated with an antibody to acetylated-lysine followed by immunoblotting with antibody to Atg3. **(B)** Translocation of GFP-Atg8 into vacuoles in 100 wild-type or *rpd3Δ* cells cultured in SD (–N) for 0.5 hour was assessed and quantified. **(C)** Duration of Atg8 puncta in wild-type and *rpd3Δ* cells cultured in SD (–N) for 1 hour. Scale bar, 2 μm. **(D)** Time course of Atg8 puncta formation in wild-type *rpd3Δ* cells cultured in SD (–N) medium. ****P** <

0.01, *****P** < 0.001 (Student's *t* test). **(E)** Wild-type and *rpd3Δ* cells cultured in SD (–N) for various time and were assessed for GFP-Atg8 cleavage. **(F)** Interactions of (top) Esa1-Myc and HA-Atg3, (middle) Epl1-Myc and HA-Atg3, and (bottom) Rpd3-Myc and HA-Atg3. Cell lysates were immunoprecipitated with antibody against HA and analyzed by means of Western blot by using an antibody against Myc. **(G)** Time course of localization of (top) Esa1-GFP and Ape1-RFP, (middle) Epl1-GFP and Ape1-RFP, and (bottom) Rpd1-GFP and Ape1-RFP. Scale bar, 2 μm. **(H)** Cells from (G) were quantified for Esa1-GFP–, Epl1-GFP–, and Rpd3-GFP–positive PAS. One hundred Ape1-RFP positive cells were counted. Error bars indicate SD.

Atg3, as the E2-like protein, together with E1-like Atg7 and E3-like Atg5-Atg12 complex covalently conjugate phosphatidylethanolamine (PE) to Atg8 (6). Atg8 lipidation reaction can be reconstituted in vitro with recombinant Atg7 and Atg3 (7). To exclude the possibility that K-R mutation may affect autophagy by directly disturbing Atg3 enzymatic activity rather than reducing Atg3 acetylation, we assayed the enzymatic activity of recombinant variants of Atg3 in an in vitro Atg8 lipidation reaction. *atg3^{K19R}*, *atg3^{K48R}*, and *atg3^{K19R-K48R}* catalyzed Atg8 lipidation, whereas *atg3^{K183R}* and *atg3^{K19R-K48R-K183R}* mediate little Atg8 lipidation (Fig. 3F). Thus, K183R mutation disrupts the enzymatic activity of Atg3, but K19R and K48R do not. Because both *atg3^{K19R-K48R}* and *atg3^{K19R-K48R-K183R}* block autophagy, we conclude that acetylation on K19 and K48 are important for regulation of autophagy, whereas K183 is important for Atg3 enzymatic activity.

Atg8 conjugation to PE requires the interaction of Atg3 with Atg8 (8). In *esa1-1*, the interaction between endogenous Atg3 and exogenous tagged Atg8 was reduced, suggesting that acetylation promotes Atg3-Atg8 interaction (Fig. 3G). The interaction between Atg8 and Atg3^{K19R-K48R} was also reduced (Fig. 3H). Thus, Esa1-mediated Atg3 acetylation appears to influence autophagy through controlling Atg3-Atg8 interaction. However, acetylation of other proteins by Esa1 may also affect autophagy.

Histone deacetylases (HDACs) participate in regulation of protein acetylation. Genetic analysis identified Rpd3 as a negative regulator of autophagy (fig. S7). Acetylation of Atg3 was increased in *rp3Δ* cells (Fig. 4A), and autophagy was accelerated (Fig. 4B and fig. S8). Time-lapse imaging revealed that the duration of Atg8 puncta was reduced in *rp3Δ* cells (Fig. 4C and fig. S9). Kinetic studies revealed that Atg3 acetylation was transiently induced by starvation (fig. S10). In contrast, Atg3 acetylation was more sustained in *rp3Δ* cells (fig. S10), suggesting that attenuation of Atg3 acetylation is mediated by Rpd3. Starvation-induced autophagy was transient in wild-type cells (Fig. 4D). However, Atg8 puncta formed rapidly but more slowly attenuated in *rp3Δ* cells (Fig. 4D), indicating that deacetylation of Atg3 by Rpd3 may contribute to the attenuation of formation of autophagosome during starvation. As a consequence of constitutive autophagosome formation, Atg8-GFP cleavage is increased in *rp3Δ* cells (Fig. 4E and fig. S11). Thus, metabolic cues appear to regulate the duration and magnitude of autophagy through temporal control of Atg3 acetylation.

Abundance of Esa1, Epl1, and Rpd3 and the enzymatic activity of Esa1 were not changed during autophagy (fig. S12). We constructed yeast strains in which hemagglutinin (HA)-Atg3 was expressed with Esa1-Myc, Epl1-Myc, and Rpd3-Myc, respectively, in physiological amount. We found weak interaction between Atg3 with Esa1 and Epl1 in yeast before starvation. Starvation transiently increased the interaction between Atg3 and Esa1 or Epl1, which peaked 1 hour after star-

vation and subsided thereafter (Fig. 4F). We also detected interaction between Atg3 and Rpd3 under nutrient-rich conditions, and starvation enhanced this interaction (Fig. 4F). We proposed that the transient acetylation of Atg3 may result from the dynamic interaction between Atg3 and acetylases. In yeast, most of Atg proteins are located on pre-autophagosomal structures (PASs) under growth or starvation conditions (9). PASs are thought to be the site for autophagosome formation and can be labeled with vacuolar hydrolases aminopeptidase I (Ape1) (10). Most Esa1, Epl1, and Rpd3 was located on the nucleus with or without nitrogen starvation (11, 12); however, we found that in starved cells a small fraction of Esa1, Epl1, and Rpd3 was located in the cytosol in the form of puncta (Fig. 4G and fig. S13). These punctas appeared to colocalize with Ape1, indicating these punctas were PASs (Fig. 4G and fig. S13). Starvation-induced transient recruitment of Esa1-GFP and Epl1-GFP to the PAS peaked 1 hour after starvation and subsided to prestarvation amount after 7 hours of starvation (Fig. 4G and fig. S13). In contrast, Rpd3-GFP was recruited to PAS on a delayed manner (Fig. 4G and fig. S13).

Acetylation is emerging as an important metabolic regulatory mechanism (13, 14) and is itself tightly regulated in response to metabolism changing (15). The TOR pathway provides one signaling pathway to couple autophagy with metabolism status. Acetylation may provide another mechanism (fig. S14).

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Supplementary Materials

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Materials and Methods
Figs. S1 to S14
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GSK3-TIP60-ULK1 Signaling Pathway Links Growth Factor Deprivation to Autophagy

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In metazoans, cells depend on extracellular growth factors for energy homeostasis. We found that glycogen synthase kinase-3 (GSK3), when deinhibited by default in cells deprived of growth factors, activates acetyltransferase TIP60 through phosphorylating TIP60-Ser⁸⁶, which directly acetylates and stimulates the protein kinase ULK1, which is required for autophagy. Cells engineered to express TIP60^{S86A} that cannot be phosphorylated by GSK3 could not undergo serum deprivation-induced autophagy. An acetylation-defective mutant of ULK1 failed to rescue autophagy in *ULK1*^{-/-} mouse embryonic fibroblasts. Cells used signaling from GSK3 to TIP60 and ULK1 to regulate autophagy when deprived of serum but not glucose. These findings uncover an activating pathway that integrates protein phosphorylation and acetylation to connect growth factor deprivation to autophagy.

Autophagy is an evolutionarily conserved cellular process by which a cell degrades its own organelles and large protein aggregates. Autophagy functions to maintain energy homeostasis under nutrient-poor conditions to allow cell survival (1–4). Unlike unicellular organisms, metazoans depend on extracellular growth factors to regulate uptake and diges-

tion of environmental nutrients. Withdrawal of growth factors or culture of cells in serum-free medium initiates autophagy (2, 5). Dysregulation of autophagy is also associated with diverse diseases, including cancers and neurodegeneration (6). Products of a series of autophagy genes (ATGs) mediate and regulate various aspects of autophagy (7–10). *ATG1* (*ULK1* and *ULK2* in mammals)

encodes a protein kinase that is inhibited by mTOR (mammalian target of rapamycin) complex 1 (mTORC1) (11–13). Inhibition of mTOR leads to activation of ULK1 and initiation of autophagy (14). ULK1 is also activated by the energy sensor kinase adenosine monophosphate-dependent protein kinase (AMPK) upon nutrient deprivation (15, 16).

We observed that the acetyltransferase TIP60 (HIV-1 Tat interactive protein, 60 kD) displayed two bands with different electrophoretic mobility (fig. S1). Mass spectrometry revealed that the upper-band TIP60 was phosphorylated on Ser⁸⁶ and Ser⁹⁰, and the lower one singly phosphorylated on Ser⁹⁰ (fig. S1). We produced a monoclonal antibody that specifically recognizes Ser⁸⁶-phosphorylated TIP60. When Ser⁹⁰ was mutated, Ser⁸⁶ was no longer phosphoryl-

ated, indicating that Ser⁹⁰ phosphorylation may be a prerequisite for Ser⁸⁶ phosphorylation (Fig. 1, A and B). The Ser⁸⁶ and Ser⁹⁰ residues are located in a conserved S/TXXS/T (where S/T represents serine/threonine, and X can be any amino acid) phosphorylation motif recognized by glycogen synthase kinase-3 (GSK3) (fig. S2) (17, 18). Co-expression of GSK3 enhanced phosphorylation of TIP60 on Ser⁸⁶, which was inhibited by treatment with GSK3 inhibitors SB216763, SB415286, or LiCl (fig. S3). In vitro phosphorylation assays demonstrated that TIP60 appears to be phosphorylated by GSK3 and cyclin-dependent kinase 1 (CDK1) through a dual-kinase mechanism (Fig. 1C and fig. S4) (19).

GSK3β is inhibited through phosphorylation at Ser⁹ in cells stimulated by growth factors. Several signaling pathways induce the inhibitory phosphorylation of GSK3, including the phosphoinositide-3-kinase (PI3K) and AKT signaling pathway, the mitogen-activated protein kinase (MAPK) cascade, and the mTOR pathway (20–24). We therefore tested whether serum deprivation would increase phosphorylation of TIP60 on Ser⁸⁶. In human colorectal cancer HCT116 cells, Ser⁸⁶ phosphorylation was increased in cells deprived of serum, concurring with a decreased phosphorylation of Ser⁹

of GSK3β and a stronger interaction between GSK3β and TIP60 (Fig. 1, D and E). Phosphorylation of Ser⁸⁶ of TIP60 was decreased in cells lacking GSK3 or in cells treated with inhibitors of GSK3, AKT, or mTOR (Fig. 1, E and G, and figs. S3 and S5A). Mouse embryonic fibroblasts (MEFs) lacking the gene for tuberous sclerosis protein 2 (*TSC2*) that have attenuated GSK3 activity (24) showed reduced phosphorylation of Ser⁸⁶ (fig. S5B).

To test whether TIP60 functions in autophagy induced by serum deprivation, we generated an HCT116 cell line (*TIP60* KD) expressing small interfering RNA (siRNA) to TIP60 (fig. S6). Depletion of *TIP60* impaired the lipidation of microtubule-associated protein 1 light chain 3 (LC3)—a marker of autophagosome formation—in cells deprived of serum (Fig. 2A). The numbers of autophagic vacuoles (AVs) and vesicles containing GFP-LC3 were also reduced (figs. S7 and S8). We next tested whether Ser⁸⁶ phosphorylation contributes to the essential role of TIP60 in autophagy induction. A MEF line in which both *TIP60* alleles are replaced by *TIP60*^{S86A} was generated (fig. S9). Similar deficiencies in autophagy induction as assessed by LC3 lipidation, p62 degradation, and autophagosome formation were observed in the *TIP60*^{S86A} cells, which could be

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Fig. 1. Phosphorylation of TIP60 by two kinases in cells deprived of serum. **(A)** Differential electrophoresis mobilities among WT-TIP60 and TIP60 mutants. **(B)** Dephosphorylation of immunoprecipitated Myc-tagged WT and S86A but not S90A-TIP60 by calf-intestinal alkaline phosphatase (CIP). **(C)** In vitro phosphorylation of TIP60. In vitro kinase assays using His-tagged WT-TIP60 or S90A-TIP60 as substrates for immunoprecipitated Myc-cyclinB1/CDK1 complexes from human embryonic kidney (HEK) 293 T cells and purified bacterially expressed GST-GSK3β. **(D)** Time course of serum-deprived HCT116 cells. Endogenous TIP60 was immunoprecipitated with antibody against TIP60. Total cell lysates (TCL) and immunoprecipitates (IP) were immunoblotted as indicated. **(E)** Modification of endogenous TIP60. Lentivirus-mediated *GSK3α* and *GSK3β* double-knockdown HCT116 cells (*GSK3*-DKD) as well as *LacZ* siRNA-expressing control cells (ctrl) were placed in serum-free medium or standard medium for 12 hours. **(F)** HCT116 cells deprived of serum were treated with dimethyl sulfoxide (DMSO) (mock) or GSK3 inhibitors SB216763 or SB415286. **(G)** GSK3 activation increased phosphorylation of TIP60 on Ser⁸⁶. HCT116 cells were placed in medium containing 20 μM inhibitor IV of AKT1 and AKT2 or 40 nM rapamycin for 16 hours. TCLs and TIP60 IPs were immunoblotted as indicated. Statistical analyses on the impacts of AKT inhibitor or rapamycin on GSK3β Ser⁹ phosphorylation were performed as described in the supplementary materials and are shown in fig. S5A.

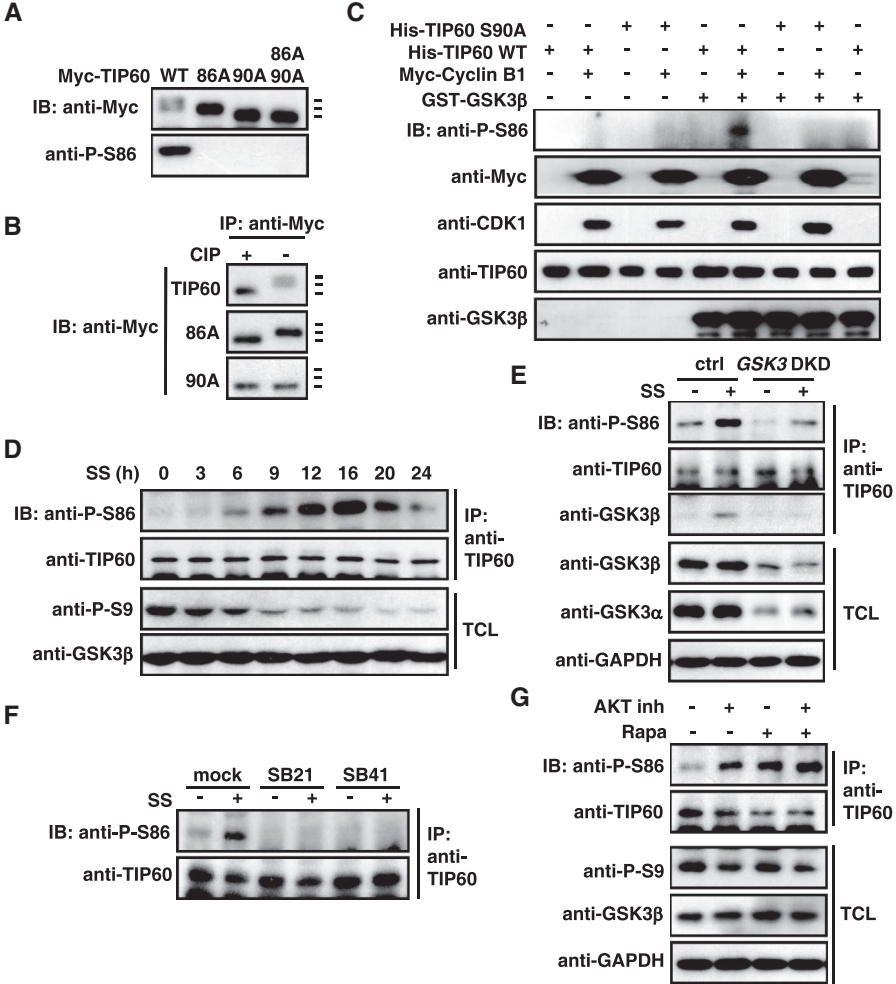


Fig. 2. TIP60 along with its Ser⁸⁶ phosphorylation is essential in serum starvation-induced autophagy. **(A)** LC3 lipidation in *TIP60* siRNA-expressing HCT116 cells (*TIP60*-KD) or the control cells (ctrl). Cells were deprived of serum for indicated times. Total proteins were extracted and immunoblotted as indicated. CQ, chloroquine. The relative amounts of LC3II were calculated from densitometry performed on immunoblots and normalized to the amount of tubulin. Data represent mean $\pm$ SEM of three independent experiments. Statistical significance was determined by analysis of variance (ANOVA); $\dagger P < 0.01$, $*P < 0.05$, $**P < 0.01$ (ANOVA followed by Tukey). **(B)** LC3 lipidation in WT and *TIP60*^{S86A} MEFs. Cytosolic proteins were then extracted and analyzed by means of immunoblotting. The graphs indicate p62 (degradation of which is a marker for autophagy activation)–to–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ratio and fold induction of LC3II. Data represent mean $\pm$ SEM of three independent experiments. $\dagger P < 0.01$ (ANOVA), $*P < 0.05$, $**P < 0.01$ (ANOVA followed by Tukey); N.S., not significant. **(C)** TIP60 from heart homogenates isolated from 3 hours after birth or prenatal rat embryos was immunoprecipitated by using antibody to TIP60, followed by immunoblotting. Each lane represents a different individual. The relative Ser⁸⁶ phosphorylation levels were calculated and normalized to total TIP60. Data are shown as mean $\pm$ SEM ($n = 4$ rats, each group). $**P = 0.0034$ (Student's *t* test).

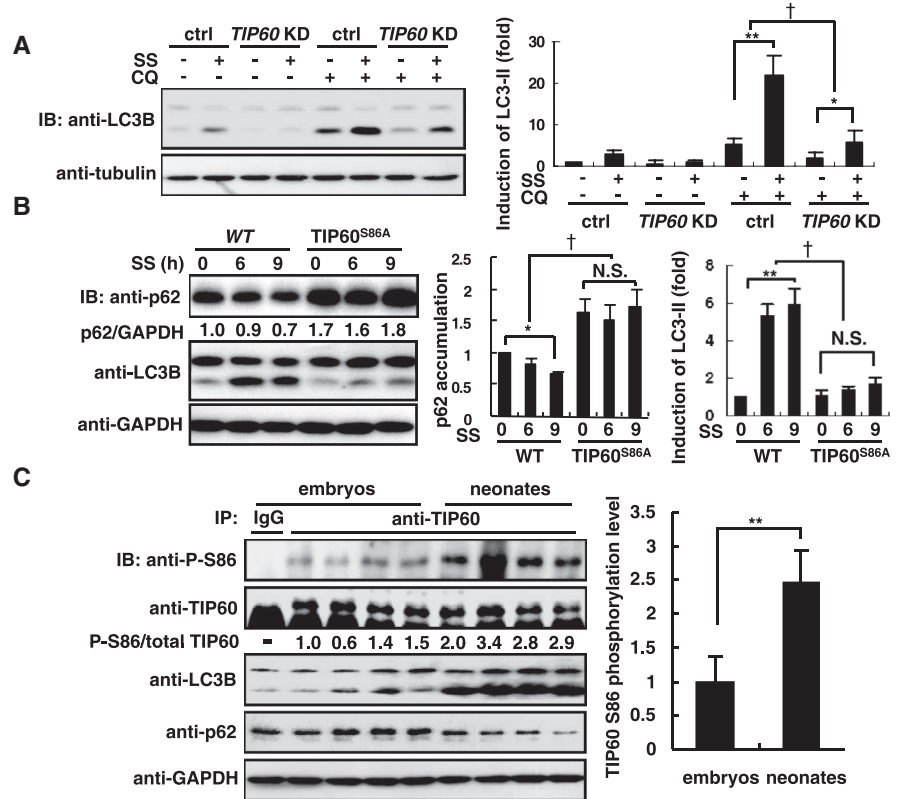


Fig. 3. Acetylation of ULK1 by TIP60 upon serum deprivation. **(A)** Association of Myc-tagged TIP60 with cotransfected HA-tagged ATGs. Protein extracts were immunoprecipitated with antibody to HA 20 hours after transfection. **(B)** Increased association between TIP60 and ULK1 in cells deprived of serum. TIP60 immunoprecipitates from HCT116 cells serum starved for 16 hours were immunoblotted as indicated. Ratio of coimmunoprecipitated ULK1 to total ULK1 was calculated. Data represent mean $\pm$ SEM of three independent experiments. $**P < 0.01$ (Student's *t* test). **(C)** Association of HA-ULK1 with Myc-TIP60. HEK293 T cells transfected with HA-ULK1 and Myc-TIP60 were treated with DMSO or 10 μ M GSK3 inhibitor SB216763 for 12 hours. **(D)** Acetylation of endogenous ULK1. *TIP60*-KD HCT116 cells and ctrl cells were serum-starved for 12 hours. Acetylated proteins were immunoprecipitated with antibody to acetylated lysine. Ratio of acetylated ULK1 to total ULK1 was calculated. Data represent mean $\pm$ SEM of three independent experiments. N.S., not significant; $**P < 0.01$ (ANOVA followed by Tukey). **(E)** Inhibition of the acetylation of ULK1 by GSK3 inhibitor. HCT116 cells were pretreated with DMSO or 10 μ M SB216763 for 4 hours before 12-hour serum starvation. Acetylated ULK1 was analyzed as in (D). **(F)** Phosphorylation-dependent activity of TIP60 toward ULK1. FLAG-TIP60 was immunoprecipitated from HEK293 T cells treated with DMSO or 10 μ M SB216763 for 12 hours; TIP60 cotransfected with HA-GSK3 β was used as a comparison. In vitro acetylation assays with purified His-ULK1 as a substrate were performed. Relative acetyltransferase activities of TIP60 toward ULK1 were calculated as the ratio of acetylated ULK1 to total ULK1. Data represent mean $\pm$ SEM of three independent experiments. $**P < 0.01$ (ANOVA followed by Tukey).

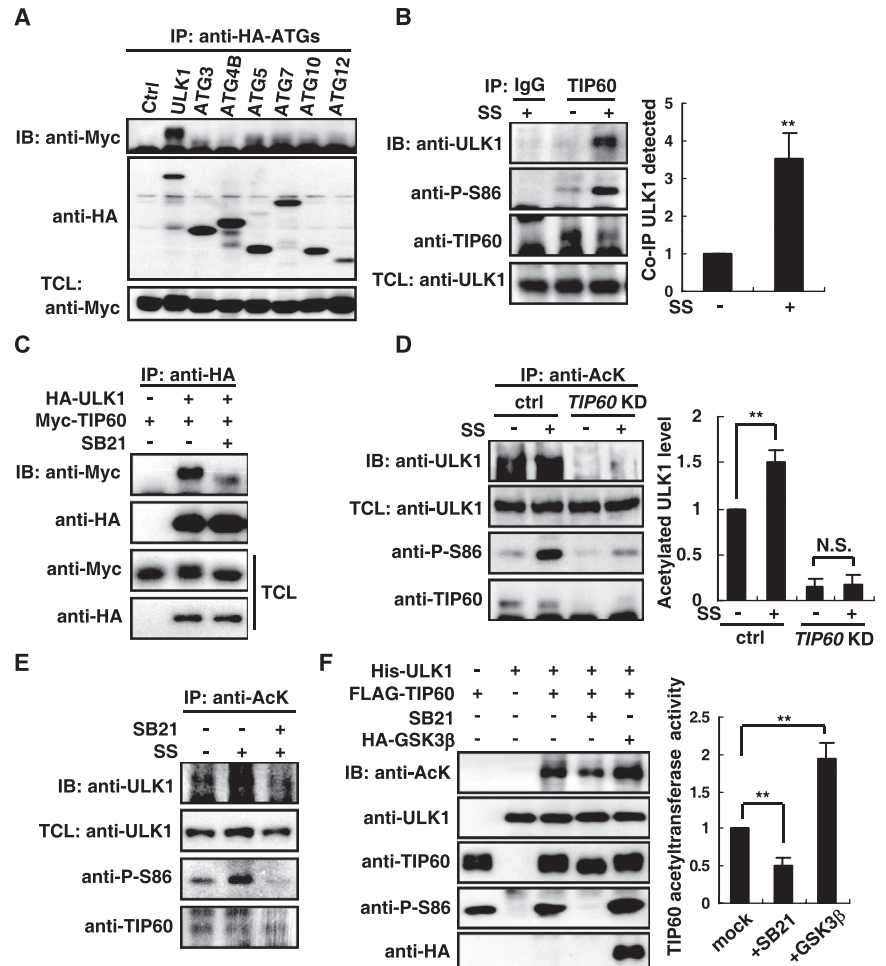
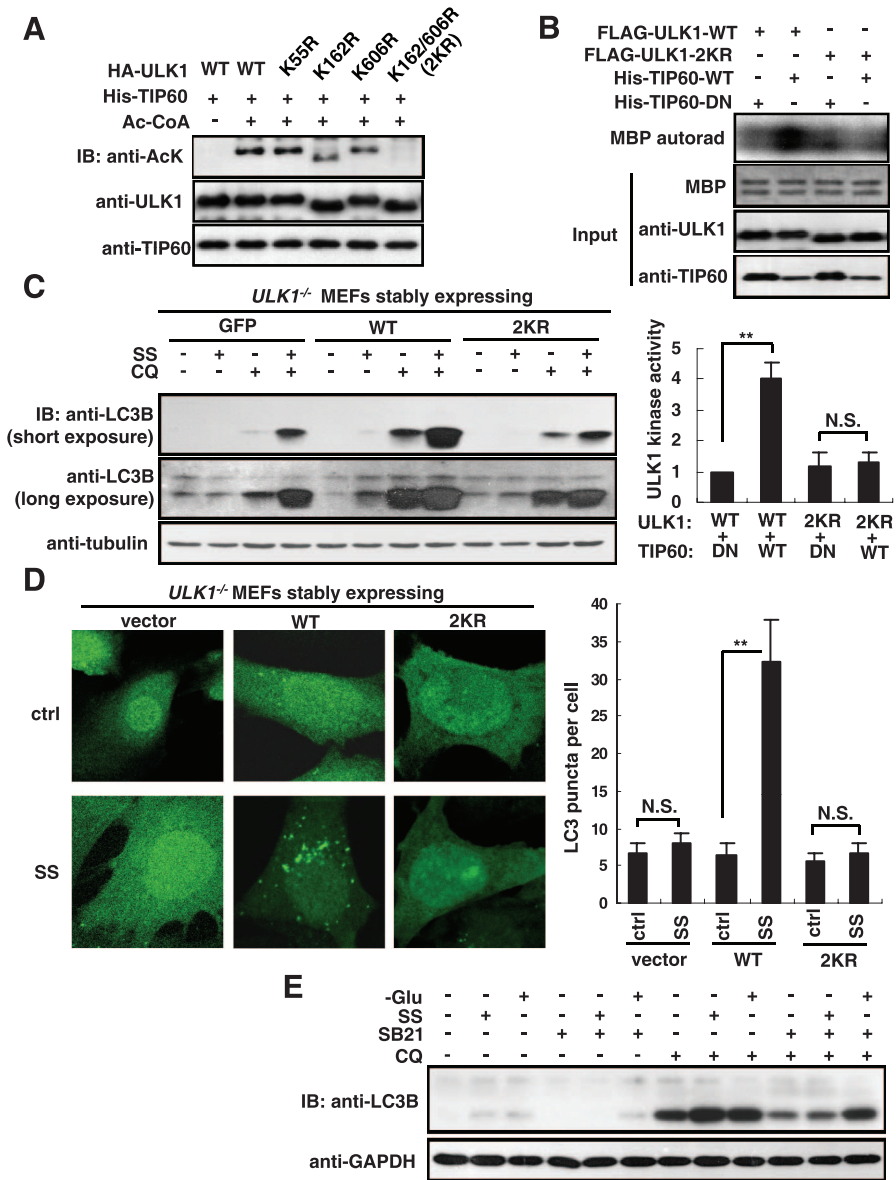


Fig. 4. Requirement of TIP60-mediated acetylation of ULK1 in serum deprivation-induced autophagy. **(A)** In vitro acetylation assays were performed to determine TIP60 acetylation of immunoprecipitated HA-tagged WT-ULK1 or its lysine-to-arginine mutants. **(B)** In vitro coupled acetylation-phosphorylation assays were performed as described in the supplementary materials, materials and methods. WT-TIP60 or acetyltransferase-dead TIP60-DN was incubated with WT-ULK1 or 2KR-ULK1 in different combinations indicated. MBP was used as the substrate for ULK1 kinase in the presence of ³²P-ATP. Data represent mean ± SEM of three independent experiments. N.S., not significant; ***P* < 0.01 (ANOVA followed by Tukey). **(C)** LC3 lipidation in *ULK1*^{−/−} MEFs stably expressing WT-ULK1 or 2KR-ULK1. Cells were serum starved for 9 hours, and total cell lysates were analyzed for LC3 lipidation by means of immunoblotting. **(D)** Autophagosome formation in *ULK1*^{−/−} MEFs. The cells were serum-starved for 9 hours after 24 hours of infection and were then fixed. LC3 positive puncta were shown as mean ± SEM of 5 random areas. N.S., not significant; ***P* < 0.01 (ANOVA followed by Tukey). **(E)** Comparison of LC3 lipidation in cells deprived of serum or glucose. HCT116 cells were pretreated with DMSO or GSK3 inhibitor SB216763 for 4 hours and then deprived of serum or glucose. Total proteins were analyzed for LC3 lipidation.



rescued by introduction of wild-type (WT) TIP60 (Fig. 2B and figs. S10 to S12). The heart of post-natal mice shows a sharp increase in autophagy activity during the first 24 hours after birth (25). We therefore measured phosphorylation of TIP60 on Ser⁸⁶ in the heart of rat embryos and neonates. Ser⁸⁶ phosphorylation was significantly increased in 3 hours after birth and subsequently reduced in 36 hours. The temporal pattern of TIP60 Ser⁸⁶ phosphorylation correlates well with that of lipidation of LC3 and degradation of p62 (Fig. 2C and fig. S13).

Because the highly conserved *ATG* genes are core components of the autophagy pathway, we tested for interaction between TIP60 and ATGs. Strong association of exogenous Myc-tagged TIP60 with hemagglutinin (HA)-tagged ULK1 (ATG1) was detected (Fig. 3A). In HCT116 cells deprived of serum, the interaction between endogenous TIP60 and ULK1 was increased (Fig.

3B). We also observed decreased interaction between exogenous TIP60 and ULK1 in cells treated with the GSK3 inhibitor SB216763 because Ser⁸⁶-phosphorylated TIP60 may preferentially bind ULK1 (Fig. 3C).

We then examined the biochemical consequences of the interaction between TIP60 and ULK1 in an in vitro acetylation assay. TIP60 acetylated ULK1 (fig. S14). In HCT116 cells deprived of serum, the amount of acetylated ULK1 was significantly increased, which was almost undetectable in cells resupplemented with growth factors such as insulin or epidermal growth factor as well as in cells expressing siRNA to TIP60 (Fig. 3D and fig. S15). GSK3 inhibitor SB216763 also led to decreased acetylation of ULK1 (Fig. 3E), indicating a requirement of TIP60 Ser⁸⁶ phosphorylation in promoting its acetyltransferase activity. TIP60 precipitated from GSK3 inhibitor-treated cells or S86A-TIP60 mutant showed com-

promised acetyltransferase activities toward ULK1; on the other hand, coexpression of GSK3 (to enhance Ser⁸⁶ phosphorylation) increased the activity (Fig. 3F and fig. S16).

We subjected acetylated ULK1 protein to mass spectrometry. Seven lysine residues were found to be candidate acetylation sites (fig. S17). We then created mutants changing these lysine residues to arginine singly or in combination. Simultaneous mutation of Lys¹⁶² and Lys⁶⁰⁶ (2KR) on ULK1 almost entirely abolished its acetylation by TIP60 in vitro (Fig. 4A). Acetylation of ULK1 by TIP60 in vitro increased its kinase activity by using myelin basic protein (MBP) as a substrate (Fig. 4B). LC3II production and the number of LC3 puncta were reduced in 2KR-expressing *ULK1*^{−/−} cells (Fig. 4, C and D).

In the absence of glucose, ULK1 is phosphorylated and activated by AMPK (15, 16). However, during 16 hours of glucose starvation

neither the inhibitory Ser⁹ phosphorylation of GSK3 β nor the phospho-Ser⁸⁶ signal was affected (fig. S18). Treatment of cells with GSK3 inhibitors decreased serum starvation–induced autophagy but not that caused by glucose deprivation (Fig. 4E). Moreover, autophagy was as effectively induced by serum starvation in *AMPK α 1/2-DKO* (double knockout) MEFs as in control MEFs (fig. S19). These results indicate that the autophagy signaling axis mediated by the GSK3 sensor kinase is distinct from that mediated by AMPK. To investigate possible interplay between mTOR-AMPK phosphorylation and TIP60 acetylation of ULK1, we examined the phosphorylation of WT-ULK1 or acetylation-deficient 2KR-ULK1 and the acetylation of phosphorylation-deficient ULK1 proteins reintroduced into the *ULK1*^{−/−} MEFs. These phosphorylation events appeared not to alter ULK1 acetylation by TIP60 or vice versa (figs. S20 and S21).

GSK3 functions in several pathways that regulate cellular homeostasis of energy and growth, including those mediated by PI3K and AKT, mTOR, and MAPK (17, 18). Under situations such as insulin or insulin-like factor withdrawal, activity of AKT is attenuated, leading to the activation of GSK3. Inhibition of mTOR by rapamycin leads to inactivation of its target kinases, including AKT, p70S6K, and p85S6K, which in turn promotes GSK3 activation (20–24). Mitogen-activated kinase signaling also inhibits GSK3 activity through the downstream kinase p90^{RSK} (22). Our present study demonstrates an autophagy-activating pathway that comprises GSK3, TIP60,

and ULK1. In this pathway, GSK3 is activated by the removal of its inhibitory Ser⁹ phosphorylation upon withdrawal of growth factors. Activated GSK3 catalyzes phosphorylation of TIP60 at Ser⁸⁶, which depends on a prior phosphorylation at Ser⁹⁰ and results in higher affinity of TIP60 for ULK1 and increased acetylation and kinase activity of ULK1. Inhibition of GSK3 can block serum but not glucose deprivation–induced autophagy, all pointing to the conjecture that glucose and growth factor deprivation use distinct machineries in autophagy induction (fig. S22). In line with an essential role of TIP60 for autophagy induction is the previous observation that *TIP60*^{−/−} mouse blastocysts fail to undergo implantation and die around embryonic day 3.5, when autophagy activity is strongly elicited for normal implantation (26, 27). The pathway we discovered may have a general role in sustaining cellular energy and metabolic substrates, considering the common sensor role of GSK3 for growth-related signaling.

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Supplementary Materials

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Materials and Methods

Figs. S1 to S22

References (28–30)

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GBP5 Promotes NLRP3 Inflammasome Assembly and Immunity in Mammals

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Inflammasomes are sensory complexes that alert the immune system to the presence of infection or tissue damage. These complexes assemble NLR (nucleotide binding and oligomerization, leucine-rich repeat) or ALR (absent in melanoma 2–like receptor) proteins to activate caspase-1 cleavage and interleukin (IL)–1 β /IL-18 secretion. Here, we identified a non-NLR/ALR human protein that stimulates inflammasome assembly: guanylate binding protein 5 (GBP5). GBP5 promoted selective NLRP3 inflammasome responses to pathogenic bacteria and soluble but not crystalline inflammasome priming agents. Generation of *Gbp5*^{−/−} mice revealed pronounced caspase-1 and IL-1 β /IL-18 cleavage defects in vitro and impaired host defense and Nlrp3-dependent inflammatory responses in vivo. Thus, GBP5 serves as a unique rheostat for NLRP3 inflammasome activation and extends our understanding of the inflammasome complex beyond its core machinery.

Inflammasomes integrate microbial- or danger-associated signals for mobilizing immunity to infection, as well as in sterile settings such as those present in gout and diabetes (1, 2). Canonical inflammasome activation involves assembly and autoproteolysis of the zymogen, procaspase-1, within multiprotein platforms containing different members of the nucleotide binding domain, leucine-rich repeat (LRR) protein

family (NLRs) or absent in melanoma 2 (AIM2)–like receptors (ALRs) (1, 2). Once activated, caspase-1 cleaves its cytokine substrates, pro-interleukin (IL)–1 β and pro-IL-18, to elicit host effector functions, prime adaptive immunity, and stimulate vicinal inflammation (1, 2).

To generate an inflammasome complex, NLRs or ALRs rely on a set of common structural motifs. These include a ligand-sensing LRR or HIN-

200 region; a nucleotide binding domain (NBD) for adenosine triphosphatase–driven oligomerization; and either a caspase activation and recruitment domain (CARD) or a pyrin domain (PYD) for engaging procaspase-1, the latter via the adapter protein ASC (apoptosis-associated speck-like protein containing a CARD). This modular design delimits the structural boundaries of inflammasome-related core proteins (1, 2).

We discovered that some NLR-like CARDs, however, are fused to guanosine 5′-triphosphatase (GTPase) domains (GDs) rather than NBD-like domains in lower organisms using genome-wide in silico screens across 91 taxa (see the supplementary text) (Fig. 1A). These GDs belonged to ancestral 65- to 73-kD guanylate binding proteins

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(GBPs), recently shown to be critical for mammalian host defense (3) (table S1 and fig. S1, A and B). This raised the possibility of functional interactions between the GBPs and NLRs/ALRs in higher species to regulate the inflammasome. We tested this idea in mammalian macrophages exposed to microbial or danger signals for each well-characterized inflammasome pathway: NLRP1, NLRP3, NLRC4, and AIM2 (1, 2). The expression of NLRP3, AIM2, and ASC is readily induced by bacterial lipopolysaccharide (LPS) or type I interferon (IFN- α/β) (4–7). Likewise, GBP expression is induced by LPS or IFN- α/β ; however, the highest levels are achievable by IFN- γ (3, 8), similar to the 47-kD immunity-related GTPases (IRGs) (9). Thus, we examined inflammasome activation in differentiated human THP-1 macrophages or C57BL/6 mouse bone marrow–derived macrophages (BMMs) primed with IFN- γ plus bacterial products to ensure that the GBPs were present at sufficient levels to assess their physiological relevance. In the case of NLRP3 inflammasomes, such priming acts together with a second signal, such as adenosine triphosphate (ATP)–induced potassium ion flux or crystal-induced phagosomal damage, to activate caspase-1. This regimen elicited dose-dependent increases in GBP expression and, as recently reported for human macrophages and keratinocytes (10, 11), heightened IL-1 β secretion and caspase-1 cleavage (Fig. 1B and figs. S2 and S3). Addition of IFN- γ also more closely

mimics the in vivo setting, where it is released from natural killer (NK) and T cells at sites of inflammation or infection (3, 8, 10, 11). Small interfering RNAs (siRNAs) against the complete human and mouse GBP families were initially screened in IFN- γ –treated macrophages given LPS/ATP, a potent combination that stimulates the NLRP3 inflammasome complex (1–3) (fig. S4). IL-1 β secretion was inhibited with siRNAs against human GBP5 and mouse Gbp5; this cross-species inhibition resembled siRNAs against human ASC and mouse Asc or the caspase-1 inhibitor Ac-YVAD-cmk (YVAD) (~50 to 80% reduction) (Fig. 1, C and D). Importantly, IL-1 β defects were not due to altered caspase-1 substrate (pro-IL-1 β) levels, IFN- γ /LPS unresponsiveness (shown by nitric oxide release), or reduced macrophage viability (fig. S5, A to C). Thus, of 17 family members tested, human GBP5 and its mouse ortholog were required for LPS/ATP-dependent IL-1 β secretion. Next, we employed additional priming agents besides LPS to delineate the inflammasome specificity of GBP5. Isolated human THP-1 and mouse J774A.1 macrophage cell lines stably expressing small hairpin RNAs (shRNAs) were used to ensure better GBP5/Gbp5 silencing; the latter was confirmed with GBP5/Gbp5-specific antibodies (figs. S4B and S5D). Notably, caspase-1/IL-1 β activation was defective for all bacterial NLRP3 priming agents tested: LPS, muramyl dipeptide (MDP), isoglutamate diaminopimelic acid (iE-

DAP), and *Salmonella typhimurium* [grown under low *Salmonella* Pathogenicity Island (SPI)-1–inducing conditions to engage NLRP3] (fig. S2D) (12). In contrast, inflammasomes containing AIM2 [directly stimulated with deoxyA;deoxyT (dA:dT)] or NLRC4 (activated with *Salmonella* flagellin in mouse macrophages) were unaffected, as were inflammasomes triggered by lysosomal disruption using the crystalline NLRP3 activator alum (13) (Fig. 1, E and F, and fig. S5, D and E). Thus, GBP5 appeared to promote NLRP3 inflammasome responses specifically to live bacteria and soluble but not crystalline priming agents in IFN- γ –treated human and mouse macrophages. To obtain unequivocal genetic evidence for this Nlrp3 selectivity and assess the contribution of Gbp5 in vivo, we generated *Gbp5*–deficient (*Gbp5*^{–/–}) mice (fig. S6). Pronounced loss of IL-1 β and IL-18 release plus caspase-1 cleavage was evident in primary *Gbp5*^{–/–} BMMs given LPS, MDP, or iE-DAP followed by ATP (Fig. 2, A and B, and fig. S7A). Similar outcomes were observed when ion flux was triggered with nigericin, ruling out ATP-P₂X₇R defects; P₂X₇R channel activity itself was comparable between genotypes (Fig. 2, A and B, and fig. S8A) (1, 2). Examination of NF- κ B (nuclear factor κ B) signaling, TNF- α (tumor necrosis factor- α), O₂[–], or NO₂[–] synthesis along with Nlrp3, Asc, procaspase-1, and pro-IL-1 β expression revealed that upstream IFN- γ /LPS signaling and induction of the core inflammasome components were still intact in *Gbp5*^{–/–}

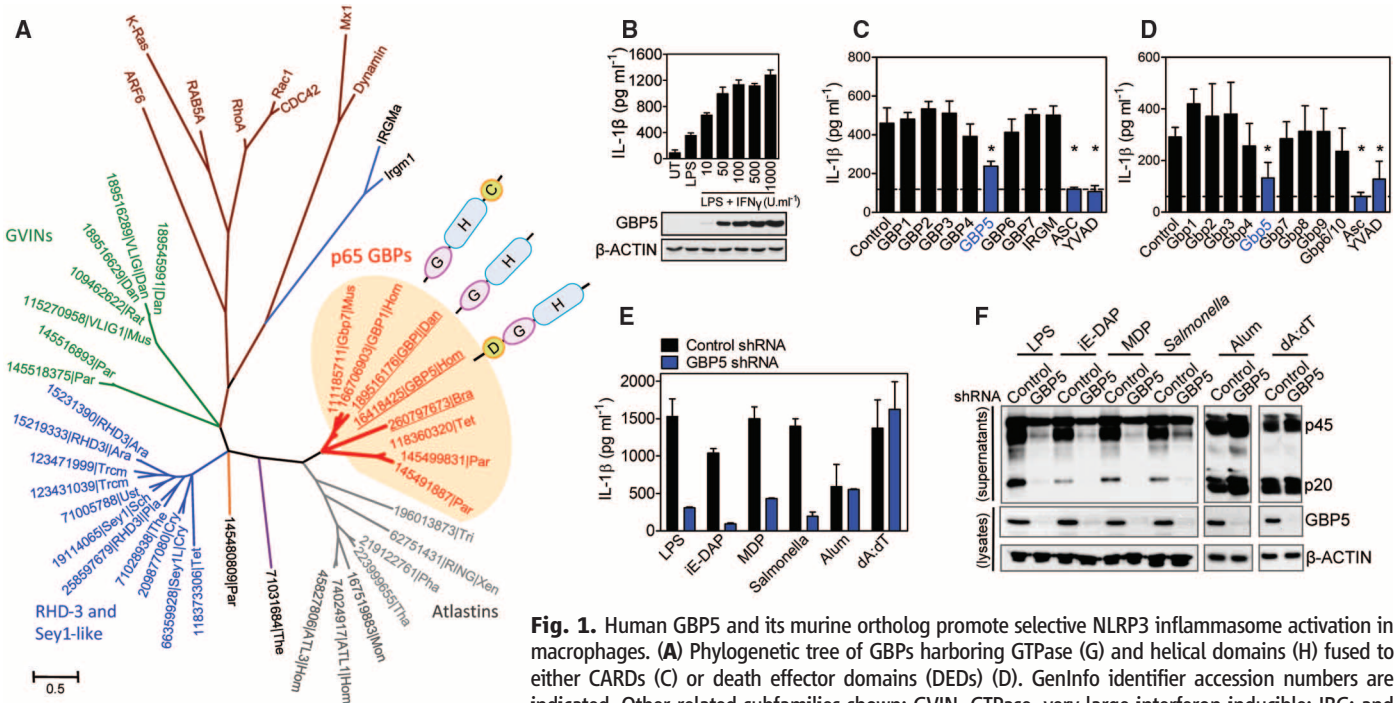


Fig. 1. Human GBP5 and its murine ortholog promote selective NLRP3 inflammasome activation in macrophages. (A) Phylogenetic tree of GBPs harboring GTPase (G) and helical domains (H) fused to either CARDs (C) or death effector domains (DEDs) (D). GenInfo identifier accession numbers are indicated. Other related subfamilies shown: GVIN, GTPase, very large interferon inducible; IRG; and RHD, root hair defective. Scale bar, 0.5 substitutions per site. (B) IL-1 β enzyme-linked immunosorbent assay (ELISA) (supernatants) and GBP5 immunoblot (cell lysates) of differentiated THP-1 cells untreated (UT) or treated with LPS alone or LPS/IFN- γ as indicated. Mean $\pm$ SD, one of three similar experiments. (C and D) IL-1 β ELISA from IFN- γ /LPS–primed THP-1 cells (C) or J774A.1 cells (D) given siRNAs or YVAD. Murine cells pulsed with ATP (5 mM) in (D). siRNAs to mouse Gbp10 also silence Gbp6 due to 99.1% nucleotide identity. Human immunity-related GTPase, M (IRGM) siRNAs served as an IRG control. Mean $\pm$ SEM, two to three experiments. *, $P < 0.05$, Student's t test. (E and F) IL-1 β and active caspase-1 (p20) from supernatants of IFN- γ –primed THP-1 cells stably expressing control or GBP5 shRNA given inflammasome priming agents. GBP5 and β -actin expression shown below. Mean $\pm$ SEM, two to three experiments.

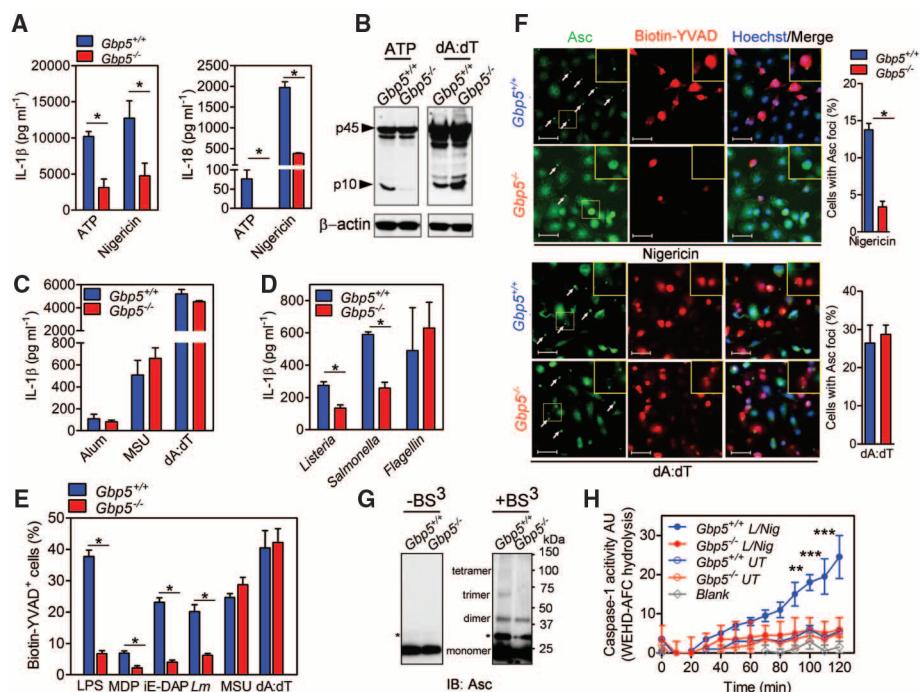


Fig. 2. Defective Nlrp3-Asc inflammasome activation and assembly in *Gbp5*^{-/-} BMMs. (**A** to **D**) Cytokines [(A), (C), and (D)] or caspase-1 (**B**) in culture supernatants of IFN- γ /LPS-primed BMMs activated with the indicated stimuli [(A) to (D)] or infected with *Listeria* or *Salmonella* (**D**). Mean $\pm$ SD; one of three to five similar experiments. (**E**) Percentage of BMMs with active caspase-1 (Biotin-YVAD⁺) (see fig. S8A). IFN- γ -primed BMMs were exposed to LPS, MDP, or iE-DAP followed by nigericin or LPS/MSU, LPS/dA:dT, or *Lm* infection. *, $P < 0.0001$, Student's t test. (**F**) Immunostaining of endogenous Asc foci (arrows) and caspase-1 in BMMs as in (**E**). Scale bar, 10 μ m. *, $P < 0.0001$, Student's t test for >1000 cells counted. Mean $\pm$ SEM from two to four experiments. (**G** and **H**) Asc multimerization (**G**) and caspase-1 activity (**H**) in inflammasomes isolated from IFN- γ /LPS-treated BMMs activated with nigericin (L/Nig) or left untreated (UT). BS³-crosslinked or native Asc (-BS³) detected by immunoblot (IB). *, Nonspecific band in (**G**). Mean $\pm$ SEM, two to three similar experiments. **, $P < 0.01$; ***, $P < 0.001$, analysis of variance (ANOVA).

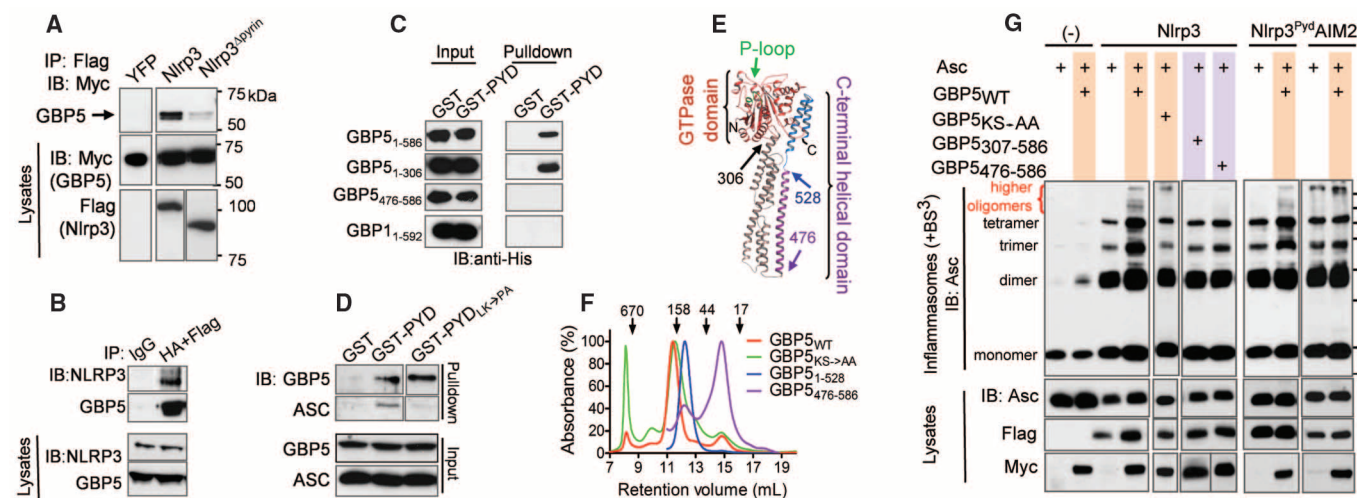


Fig. 3. Tetrameric GBP5 binds Nlrp3 to promote Asc assembly. (**A**) Coimmunoprecipitation and immunoblot of Myc-GBP5 and Flag-tagged Nlrp3 [intact or pyrin-deleted (Δ pyrin)] from HEK293 cells. Yellow fluorescent protein (YFP) served as a nonspecific control. One of three similar experiments. (**B**) Immunoblot of endogenous NLRP3 immunoprecipitated by hemagglutinin-Flag-GBP5 stably expressed in THP-1 cells. One of two similar experiments. (**C** and **D**) GST pull-down of recombinant GBP5 and GBP1 (**C**) or native GBP5 and ASC (**D**) from IFN- γ -treated THP-1 cells. GST, GST-PYD (GST-NLRP3₁₋₁₁₄),

or GST-PYD_{LK $\rightarrow$ PA} used as bait. One of three experiments shown. (**E**) GBP5 homology model depicting N-terminal GTPase (nucleotide-binding P-loop) and C-terminal α -helical domains based on the GBP1 crystal structure (Protein DataBank code 1F5N). (**F**) Size-exclusion chromatographs of recombinant GBP5 proteins. Arrows, molecular weight (kD) calibration. (**G**) Asc oligomerization assay in HEK293 cells transfected with Myc-GBP5 variants plus Flag-tagged NLRP3, NLRP3^{PYD}, or AIM2. Lysate IB of expressed proteins below. One of three similar experiments.

Both mitochondrial reactive oxygen species and membrane potential have been proposed to contribute to this nonlysosomal pathway (7, 19, 20). No differences, however, were observed for either parameter between *Gbp5*^{+/+} and *Gbp5*^{-/-} BMs (fig. S8D). Thus, Gbp5 likely operates more proximal to the caspase-1 complex itself.

We assayed inflammasome complex formation by tracking the Nlrp3 adaptor, Asc, which oligomerizes as part of this complex to form a single large (1 to 2 μ m) perinuclear focus per cell (12, 21). Such foci assemble in a stimulus- and NLR-dependent but caspase-1-independent manner (12, 21). IFN- γ -primed *Gbp5*^{-/-} BMs revealed almost complete loss (~85%) of Asc foci after LPS/nigericin treatment for engaging the Nlrp3 inflammasome, whereas Aim2 inflammasome activation with dA:dT was unaffected (Fig. 2F). Asc oligomerization and caspase-1 activity were essentially absent within enriched inflammasome fractions isolated from *Gbp5*^{-/-} macrophages given LPS/nigericin (Fig. 2, G and H). Thus, Gbp5 promotes Nlrp3-Asc inflammasome assembly to activate caspase-1 under native conditions.

This assembly may require interactions of Gbp5 with Nlrp3. Mouse Nlrp3 captured Gbp5 in a PYD-dependent manner (other PYD/CARD-containing inflammasome proteins Nlrp1, Nlrp4, Aim2, Asc, or procaspase-1 failed to do so) and human GBP5 retrieved endogenous NLRP3 from IFN- γ -treated THP-1 cells (Fig. 3, A and B, and fig. S9, A to C). Such interactions were specific; a recombinant glutathione *S*-transferase (GST)-fused PYD of NLRP3 (NLRP3^{PYD}) bound GBP5 via its GTPase domain (GBP5₁₋₃₀₆) but did not bind its closest analog, GBP1 (Fig. 3C). NLRP3^{PYD} also retrieved endogenous human GBP5 and ASC via distinct subdomains, as shown using an L22P, K23A mutant (GST-NLRP3^{PYD}_{LK→PA}) that captured native GBP5 but not ASC (Fig. 3D). Interaction of GBP5 with NLRP3^{PYD} led to assembly of scaffolds around an inner Asc core as seen by fluorescent microscopy (fig. S9, D to F).

Once bound, how does GBP5 promote NLRP3 assembly? The forerunner of the GBP family, GBP1, displays GTPase-dependent tetramerization (22), a process that could help drive complex formation. We built an energetically favored GBP5 model using the GBP1 crystal structure to assign truncation/mutation sites for examining self-assembly (Fig. 3E). Recombinant GBP5 and a GTPase-deficient mutant (GBP5_{KS→AA}) both formed tetramers (Fig. 3F and fig. S10). This GTPase-independent tetramerization required the last 62 amino acids, which by modeling fold back on its G domain (Fig. 3, E and F). Removal of this region (GBP5₁₋₅₂₈) yielded GBP5 dimers, while a coiled-coil domain within it also dimerized (GBP5₄₇₆₋₅₈₆) (Fig. 3, E and F). Hence a “dimer of dimers” model could account for GBP5 tetramer assembly to stimulate Nlrp3-Asc oligomerization seen in wild-type but not *Gbp5*^{-/-} cells (Fig. 2, G and H).

We tested this idea by reconstituting Nlrp3-Asc complexes in human embryonic kidney

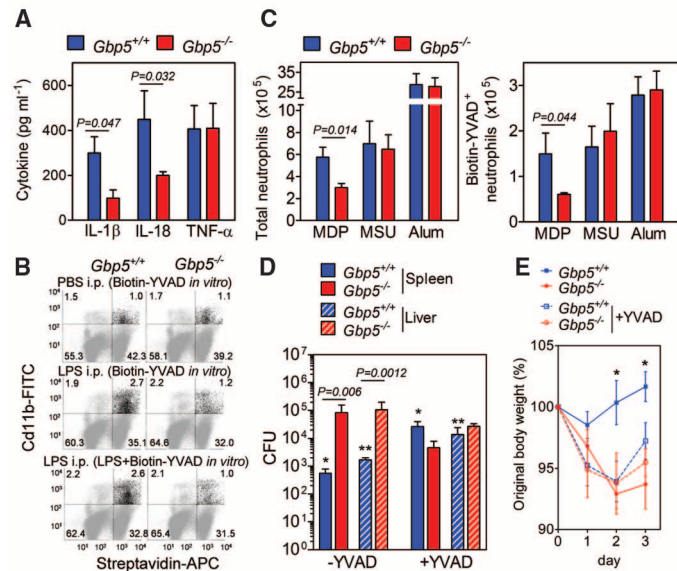


Fig. 4. Gbp5 promotes Nlrp3-dependent responses in vivo. (A) Serum cytokines 3 hours after LPS injection [30 mg per kg of weight intraperitoneally (ip); *n* = 6 to 7 mice per group]. Mean $\pm$ SEM from two to three experiments. *P* values, Student's *t* test. (B) Flow cytometric staining of active caspase-1 [Biotin-YVAD-Streptavidin-Allophycocyanin (APC)] in CD11b⁺ splenocytes 3 hours after LPS or phosphate-buffered saline as control. *Gbp5*^{-/-} cells were restimulated with LPS to ensure that they were not tolerized to LPS in vivo. (C) Caspase-1 activation in peritoneal exudate neutrophils (Ly6G⁺) of mice (*n* = 3 to 8 per group) injected intraperitoneally with different Nlrp3 agonists. Mean $\pm$ SEM, two to five experiments. *P* values, Student's *t* test. (D and E) Mice orally infected with *Listeria* and in two groups treated with YVAD (1 mg/day ip) before assaying organ bacterial burdens [colony-forming units (CFU)] (D); and body weight (E) 3 days later (mean $\pm$ SEM; *n* = 4 to 9 per group). *P* values: two-tailed Mann-Whitney test in (D); ANOVA (*P* < 0.001) at days 2 and 3 in (E). Similar *Listeria* colonization (~1000 CFU in spleen and liver; day 1) for both groups in two experiments.

(HEK) 293 cells lacking each of these components. Asc multimerization was modest in the presence of either NLRP3 or NLRP3^{PYD} but greatly enhanced if tetrameric GBP5 or GBP5_{KS→AA} was added (Fig. 3G). Asc assembly was abolished when the tetramerization mutants, GBP5₃₀₇₋₅₈₆ and GBP5₄₇₆₋₅₈₆, were used. Replacement of NLRP3 by the Asc-binding ALR, AIM2, also made this process GBP5-independent (Fig. 3G). Thus, tetrameric GBP5 promotes Asc oligomerization specifically through its interaction with NLRP3.

Finally, we examined the impact of Gbp5 on Nlrp3-Asc assembly for caspase-1 activation and cytokine secretion in vivo. Three inflammatory and infection models were used. First, LPS-induced sepsis (23, 24) revealed that *Gbp5*^{-/-} animals had 60 to 75% reduction in serum IL-1 β and IL-18 but similar TNF- α levels to wild-type mice, confirming inflammasome specificity (Fig. 4A). This coincided with loss of active caspase-1-expressing (Biotin-YVAD⁺) *Gbp5*^{-/-} CD11b⁺ macrophages in target organs such as the spleen (Fig. 4B and fig. S11, A to C). Second, in a caspase-1-dependent model of peritonitis (13, 25), *Gbp5*^{-/-} mice recruited significantly fewer Biotin-YVAD⁺ neutrophils after injection with MDP but not crystalline MSU or alum (Fig. 4C). Hence the selective inflammasome defects seen for soluble Nlrp3 priming agents (LPS and MDP) in vitro also operated in vivo. Third, orogastric chal-

lenge with *L. monocytogenes*, a natural food-borne pathogen detected in part by Nlrp3-Asc inflammasomes and requiring caspase-1 for resistance (15, 16, 26), led to higher bacterial burdens, discernible weight loss, and 50 to 80% fewer Biotin-YVAD⁺ CD11b⁺ cells in mesenteric lymph nodes of *Gbp5*^{-/-} mice, confirming defects in caspase-1 activation in vivo (15, 16, 26) (Fig. 4, D and E, and fig. S11D). Inhibiting this caspase-1 activation in wild-type mice with daily YVAD injections led to susceptibility resembling *Gbp5*^{-/-} mice, whereas the same treatment failed to render the latter group more susceptible given that they are already compromised for this pathway (Fig. 4, D and E). Thus, Gbp5 promotes caspase-1-mediated protection against oral *Listeria* infection and Nlrp3-dependent inflammatory responses in vivo.

Collectively, our findings identify GBP5 as a unique activator of NLRP3-Asc assembly to live bacteria and their cell wall components but not crystalline agents or double-stranded DNA (fig. S12). How GBP5 elicits this selective response may involve spatial segregation of soluble versus crystalline cytosolic signals, events that could affect the recently discovered noncanonical inflammasome pathway as well (24). Moreover, results from *Gbp5*^{-/-} mice suggest that a search for human GBP5 mutations—like NLRP3 variants in Crohn's colitis or autoinflammatory syndromes (1, 2)—could have important implications for human health.

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Supplementary Materials

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The Inhibitory Receptor PD-1 Regulates IgA Selection and Bacterial Composition in the Gut

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Immunoglobulin A (IgA) is essential to maintain the symbiotic balance between gut bacterial communities and the host immune system. Here we provide evidence that the inhibitory co-receptor programmed cell death-1 (PD-1) regulates the gut microbiota through appropriate selection of IgA plasma cell repertoires. PD-1 deficiency generates an excess number of T follicular helper (T_{FH}) cells with altered phenotypes, which results in dysregulated selection of IgA precursor cells in the germinal center of Peyer's patches. Consequently, the IgAs produced in PD-1-deficient mice have reduced bacteria-binding capacity, which causes alterations of microbial communities in the gut. Thus, PD-1 plays a critical role in regulation of antibody diversification required for the maintenance of intact mucosal barrier.

The primary function of immunoglobulin A (IgA) is to maintain homeostasis at mucosal surfaces. Intestinal IgA production occurs via both T helper cell-dependent and independent pathways (1). The diversification of IgA repertoire by somatic hypermutation (SHM), however, takes place mostly in specialized microenvironments called germinal centers (GCs), in which B cell interaction with T follicular helper (T_{FH}) cells induces the expression of activation-induced cytidine deaminase (AID) (2, 3). T_{FH} cells express high amounts of the inhibitory co-receptor programmed cell death-1 (PD-1) (4).

PD-1 deficiency leads to species-specific, antibody-mediated autoimmune diseases (5–7). Note that the incidence of diseases in PD-1-deficient mice varies among mouse colonies and depends on AID (8).

We investigated whether PD-1 regulates microbial communities and IgA production in the gut. Although the total number of bacteria cultured from the lumen of the small intestine was comparable between PD-1-deficient mice (*Pdcd1*^{−/−}) and wild-type (WT) mice (4.8 × 10⁸ and 4.7 × 10⁸ bacteria per g of intestinal content, respectively), *Pdcd1*^{−/−} mice had a 93 to 95% reduction in the number of anaerobic bacteria compared with WT mice (average 2.86 × 10⁸ and 0.18 × 10⁸ in WT and *Pdcd1*^{−/−} mice, respectively) (Fig. 1A). The total numbers of “healthy” bacteria, such as *Bifidobacterium* and *Bacteroides* (9) were not detectable or markedly reduced in *Pdcd1*^{−/−} mice. In contrast, bacteria of the *Enterobacteriaceae* family, which were minor representatives in the WT mice, were increased about 400-fold in *Pdcd1*^{−/−} mice. These results were confirmed by 16S ribosomal RNA (rRNA) gene pyrosequencing of cecal contents

(fig. S1A). Thus, PD-1 deficiency perturbs the balance of bacterial communities in the gut.

The frequencies and the absolute numbers of IgA-producing cells in the lamina propria (LP) were comparable in *Pdcd1*^{−/−} and WT mice (fig. S1, B, C, and F). Flow cytometric analyses of fecal bacteria revealed, however, that the proportion of bacteria coated with IgA (bound IgAs) was reduced in *Pdcd1*^{−/−} mice (10) (Fig. 1B). In contrast, the concentration of free IgA in intestinal secretions was higher in *Pdcd1*^{−/−} than in WT mice (Fig. 1C). To assess the features of LP IgA, we sequenced the immunoglobulin heavy chain (IgH) genes in single sorted IgA-producing cells. Both WT and *Pdcd1*^{−/−} mice had a diverse IgA repertoire, yet *Pdcd1*^{−/−} mice had an enrichment of IgA-producing cells with the IgH locus belonging to non-V_H1 family genes (Fig. 1D). This difference in the IgA repertoire may be the result of the altered composition of the microflora in *Pdcd1*^{−/−} mice. Indeed, as signs of antigen-mediated selection in their IgH genes, about 90% of the sequences from WT and *Pdcd1*^{−/−} mice had mutations and high ratios of replacement (R) to silent (S) mutations in complementarity-determining region 1 (CDR1) and CDR2, compared with those in framework regions 1 to 3 (FWR1-3) (fig. S1, D and E). However, the calculated affinity maturation index (11, 12) was lower in IgA-producing cells from LP of *Pdcd1*^{−/−} mice (Fig. 1E). Together, the results indicate that alterations of IgA compartment in *Pdcd1*^{−/−} mice have an impact on symbiotic relations between host and commensal bacteria in the gut.

The altered repertoire of the IgA plasma cells in gut could result from changes in the turnover of IgAs at their inductive or residential places. Most of the IgA⁺ B cells are generated in the GCs of Peyer's patches (PPs) and arrive at the LP as plasmablasts [B220⁺ IgA⁺ MHCII⁺ (major histocompatibility class II-positive), hereafter, PBs] (13). In the LP, PBs down-regulate the B cell receptor and MHCII and differentiate into plasma cells (B220[−] IgA⁺ MHCII^{low} CD138⁺, hereafter, PCs) (fig. S1F). The proliferation of

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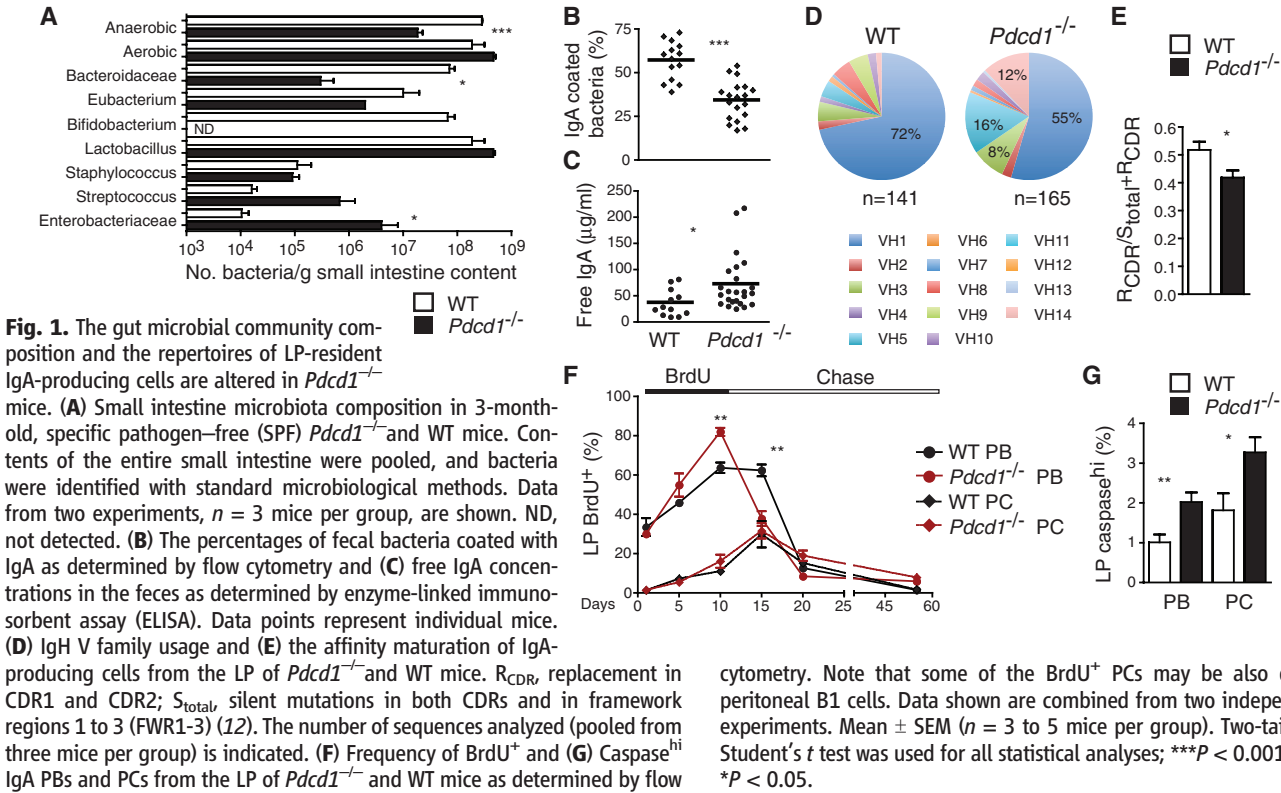


Fig. 1. The gut microbial community composition and the repertoires of LP-resident IgA-producing cells are altered in *Pdc1*^{-/-} mice. **(A)** Small intestine microbiota composition in 3-month-old, specific pathogen-free (SPF) *Pdc1*^{-/-} and WT mice. Contents of the entire small intestine were pooled, and bacteria were identified with standard microbiological methods. Data from two experiments, *n* = 3 mice per group, are shown. ND, not detected. **(B)** The percentages of fecal bacteria coated with IgA as determined by flow cytometry and **(C)** free IgA concentrations in the feces as determined by enzyme-linked immunosorbent assay (ELISA). Data points represent individual mice. **(D)** IgH V family usage and **(E)** the affinity maturation of IgA-producing cells from the LP of *Pdc1*^{-/-} and WT mice. R_{CDR}, replacement in CDR1 and CDR2; S_{total}, silent mutations in both CDRs and in framework regions 1 to 3 (FWR1-3) (12). The number of sequences analyzed (pooled from three mice per group) is indicated. **(F)** Frequency of BrdU⁺ and **(G)** Caspase^{hi} IgA PBs and PCs from the LP of *Pdc1*^{-/-} and WT mice as determined by flow

cytometry. Note that some of the BrdU⁺ PCs may be also derived from peritoneal B1 cells. Data shown are combined from two independent sets of experiments. Mean ± SEM (*n* = 3 to 5 mice per group). Two-tailed unpaired Student's *t* test was used for all statistical analyses; ****P* < 0.001; ***P* < 0.01, **P* < 0.05.

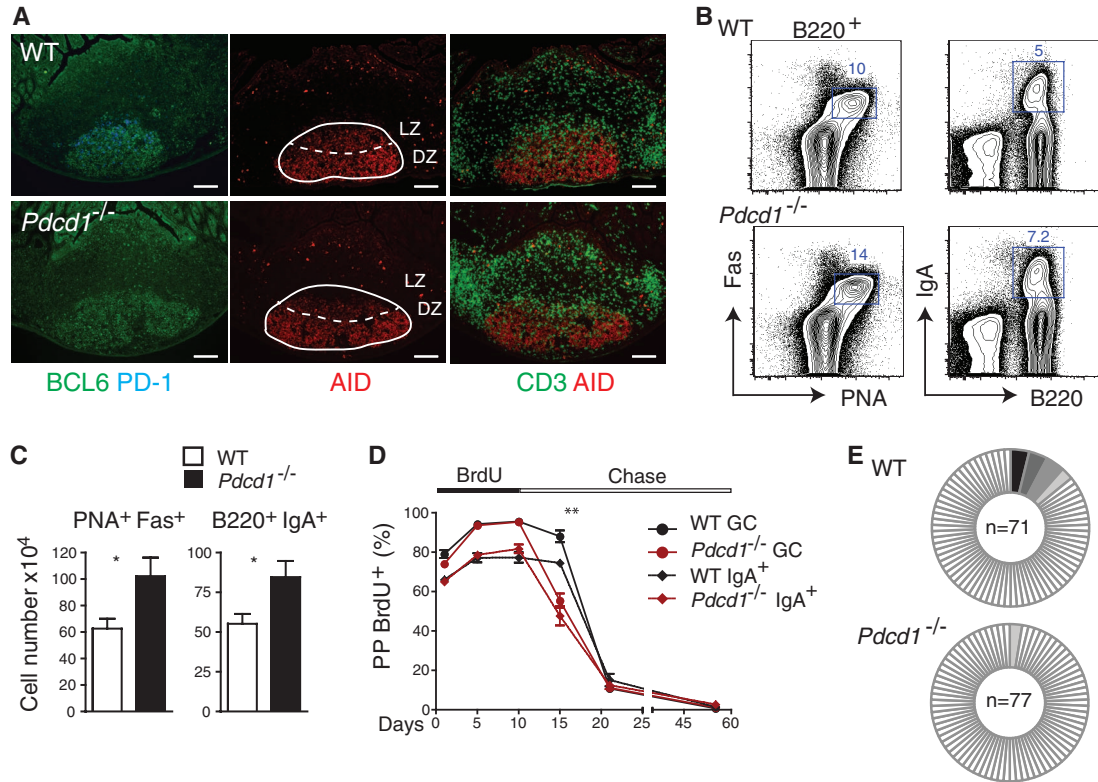


Fig. 2. Increased numbers and enhanced dynamics of GC and IgA⁺ B cells in PPs of *Pdc1*^{-/-} mice. **(A)** Representative sections of the PPs and **(B)** flow cytometric profiles of PP cells from WT and *Pdc1*^{-/-} mice stained as indicated to reveal the structure and characteristics of GCs. Scale bars, 100 μm. LZ (light-zone AID^{low}) and DZ (dark-zone AID^{hi}) are shown. **(C)** Absolute numbers of indicated B cell populations isolated from PPs of WT and *Pdc1*^{-/-} mice. Means ± SEM (*n* = 16 mice per group). **(D)** Frequency of BrdU⁺ GC B cells (PNA⁺Fas⁺) and B220⁺IgA⁺ B

cells from PPs of *Pdc1*^{-/-} and WT mice as determined by flow cytometry. Data are combined from two independent sets of experiments. Means ± SEM (*n* = 3 to 5 mice per group). **(E)** Representative charts of clonal diversity, calculated as frequency of transcripts of a given in-frame V_H(N)-D-(N)-J_H rearrangement of GC IgA⁺ B cells in PPs of *Pdc1*^{-/-} and WT mice. The number of productive sequences compared is indicated. Two-tailed unpaired Student's *t* test was used for all statistical analyses; ***P* < 0.01; **P* < 0.05.

IgA-producing cells in LP was assessed using fluorescent ubiquitination-based cell-cycle indicator (Fucci) mice, in which the cells in S-G₂-M phase of the cell cycle are fluorescently marked (14). Similar to humans, most of the proliferating cells were PBs (13, 15), and the frequency of PBs in the cell cycle was comparable for *Pdcd1*^{-/-} mice and WT mice (fig. S1F).

We next determined the turnover of IgA-producing cells in LP based on 5-bromo-2'-deoxyuridine (BrdU) incorporation. Mice were continuously fed BrdU for 10 days, and then BrdU was removed from their drinking water. Ten days after the initiation of BrdU administration, there were significantly more BrdU⁺ PBs in the LP of *Pdcd1*^{-/-} mice than in those of WT mice (Fig. 1F). Five days after the removal of BrdU, however, the frequency of BrdU⁺ PBs was reduced by half in *Pdcd1*^{-/-} mice, whereas the frequency of BrdU⁺ PBs in WT mice remained unchanged (Fig. 1F). In contrast, the frequency of BrdU-labeled PCs did not differ significantly between *Pdcd1*^{-/-} and WT mice (Fig. 1F). The higher turnover of *Pdcd1*^{-/-} PBs may be the

result of increased apoptosis. Cell death was assessed by detection of caspase activation. Compared with WT mice, we observed a significant increase in frequency of caspase^{hi} PBs and PCs in the LP of *Pdcd1*^{-/-} mice, but this diminished after antibiotic treatment (Fig. 1G and fig. S1G). Together, these results indicate enhanced commensal-driven turnover of IgA-producing cells in LP of *Pdcd1*^{-/-} mice.

Next, we examined the PPs of *Pdcd1*^{-/-} mice. Compared with WT mice, *Pdcd1*^{-/-} mice had significantly more peanut agglutinin-positive (PNA⁺) Fas⁺ GC B cells and IgA⁺ B cells in PPs (Fig. 2, A to C). Although we observed a modest increase in the frequency of light-zone B cells in the PP GCs of *Pdcd1*^{-/-} mice (16) (fig. S2A), quantitative real-time fluorescence polymerase chain reaction analyses of several important B cell markers for dark-zone and light-zone GC B cells did not show significant differences between *Pdcd1*^{-/-} mice and WT mice (fig. S2B). A comparable proportion of IgA⁺ GC cells from *Pdcd1*^{-/-} and WT mice were in cell cycle, as assessed by Fucci or a 12-hour BrdU pulse test

(17) (fig. S2, A and C). The pulse-chase BrdU experiment revealed considerable differences in dynamics of IgA GCs in *Pdcd1*^{-/-} mice, however. Namely, in both *Pdcd1*^{-/-} and WT mice, the frequency of GC and IgA⁺ B cells that incorporated BrdU reached a plateau of 95 and 80%, respectively, 5 days after the initiation of BrdU labeling (Fig. 2D). However, 5 days after the removal of BrdU, the frequency of labeled GC and IgA⁺ B cells was reduced by half in *Pdcd1*^{-/-} mice, whereas most of the BrdU⁺ cells remained in the PPs in WT mice (Fig. 2D). The significant drop in BrdU⁺ cells in PPs of *Pdcd1*^{-/-} mice could be the result of increased cell death in situ. However, there were no differences in the frequency of caspase^{hi} GCs and IgA⁺ cells for WT and *Pdcd1*^{-/-} mice (fig. S2D). The data suggest that the increased turnover of IgA⁺ B cells in PPs of *Pdcd1*^{-/-} mice may be because they pass through the GCs more rapidly. Also, the increased number of GC B cells in *Pdcd1*^{-/-} mice likely results from more inflow of B cells into GCs. Indeed, the frequency and numbers of IgD⁺ cells with an early GC phenotype

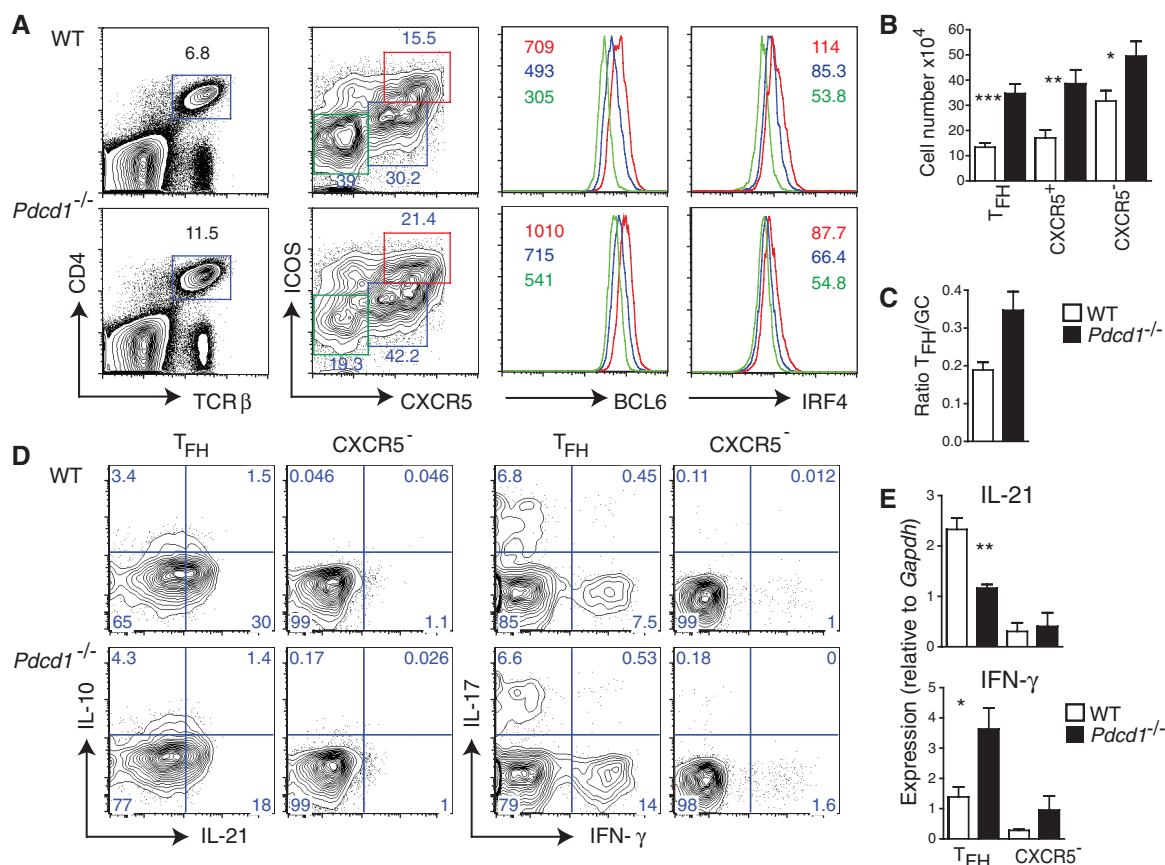


Fig. 3. Expansion of activated T cells and skewed cytokine profiles of T_{FH} cells in PP GCs of *Pdcd1*^{-/-} mice. (A) Representative flow cytometric profiles and plots of PP cells from WT and *Pdcd1*^{-/-} mice stained for the indicated markers. Numbers in the graphs indicate the geometric mean fluorescence intensity of BCL6 and IRF4 in the corresponding color-coded T cell subset gates. (B) Absolute numbers of major T cell populations and (C) the ratio of T_{FH} cells to GC B cells in the PPs of *Pdcd1*^{-/-} and WT mice. Means ± SEM (*n* =

at least 5 mice per group). (D) Flow cytometric profiles and (E) mRNA expression of indicated cytokines in PP T cell subsets. Numbers represent percentage of cells in the quadrants or gates. Relative amounts of mRNA normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown. Means ± SEM of at least two independent experiments. Two-tailed unpaired Student's *t* test was used for all statistical analyses; ****P* < 0.001; ***P* < 0.01, **P* < 0.05.

(B220⁺IgD⁺IgA⁺GL7⁺Fas⁺CCR6⁺) (18) were higher in PP of *Pdcd1*^{-/-} mice (fig. S2, E and F). Taken together, our results suggest that PD-1 deficiency is associated with an increased import and export of B cells into the GC. Such an abbreviated transit time likely results in impaired clonal selection and expansion of IgA⁺ B cells in GCs of PPs, as shown by the reduction of the frequency of clonally related IgA sequences [identical V_H(N)-D(N)-J_H where V_H is the variable region of the immunoglobulin heavy chain, N is a random nucleotide, D is the diversity region, and J_H is the joining region of the immunoglobulin heavy chain] in *Pdcd1*^{-/-} mice compared with WT mice (Fig. 2E).

We then asked why PD-1 deficiency causes such changes in the dynamics of GC B cells in PPs. It is well established that the number and activation status of T cells—and T_{FH} cells in particular—play a fundamental role in shaping GC biology. In fact, B cells compete for T cell help before entry into GCs, as well as within GCs, and deregulation of T_{FH} cells leads to inappropriate GC B cell selection and humoral autoimmunity (16–19). Thus, we assessed T_{FH} cells in the PPs of *Pdcd1*^{-/-} mice. The frequency and absolute number of CD4⁺ T cells, including CXCR5^{hi}ICOS^{hi} (high levels of ICOS, protein-inducible costimulator) T_{FH} cells, were significantly increased in PPs of *Pdcd1*^{-/-} mice (Fig. 3, A and B, and Fig. 2A). The ratio of T_{FH} to GC B cells in *Pdcd1*^{-/-} mice was twice as high as that in WT mice (Fig. 3C). The expression of BCL6, a transcription factor required for T_{FH} differentiation, was higher in all CD4⁺ T cell subsets, including the T_{FH} cells in PPs of *Pdcd1*^{-/-} mice (Fig. 3A). In contrast, the expression of interferon regulatory factor 4 (IRF4), which is required for the production of interleukin-21 (IL-21), the cytokine that promotes growth and differentiation of IgA⁺ B cells into PCs (19, 20), was reduced in T_{FH} as well as CXCR5^{hi}ICOS^{int} cells from *Pdcd1*^{-/-} mice. Indeed, T_{FH} cells from *Pdcd1*^{-/-} mice produced less IL-21 compared with those from WT mice, as previously reported (21) (Fig. 3, D and E). Furthermore, the proportion of T_{FH} cells producing interferon-γ (IFN-γ) was increased in PPs of *Pdcd1*^{-/-} mice.

In order to better characterize how T_{FH} cells may contribute to the altered GC responses observed in *Pdcd1*^{-/-} mice, we decided to evaluate the ability of PD-1-deficient T_{FH} cells to support IgA generation in gut in vivo. Thus, PP CXCR5^{hi}ICOS^{hi} T_{FH} cells were isolated from *Pdcd1*^{-/-} and WT mice and were adoptively transferred into T cell-deficient (lacking the CD3ε subunit, *Cd3e*^{-/-}) mice. Injection of T_{FH} cells from both WT and *Pdcd1*^{-/-} mice into *Cd3e*^{-/-} mice reconstituted the GC B cells and B220⁺IgA⁺ B cells in PPs (Fig. 4, A and B). However, *Cd3e*^{-/-} mice transferred with PD-1-deficient T_{FH} cells generated very few IgA-secreting cells in the LP compared with mice injected with WT T_{FH} cells (Fig. 4, A to C). The few IgAs induced by *Pdcd1*^{-/-} T_{FH} cells had

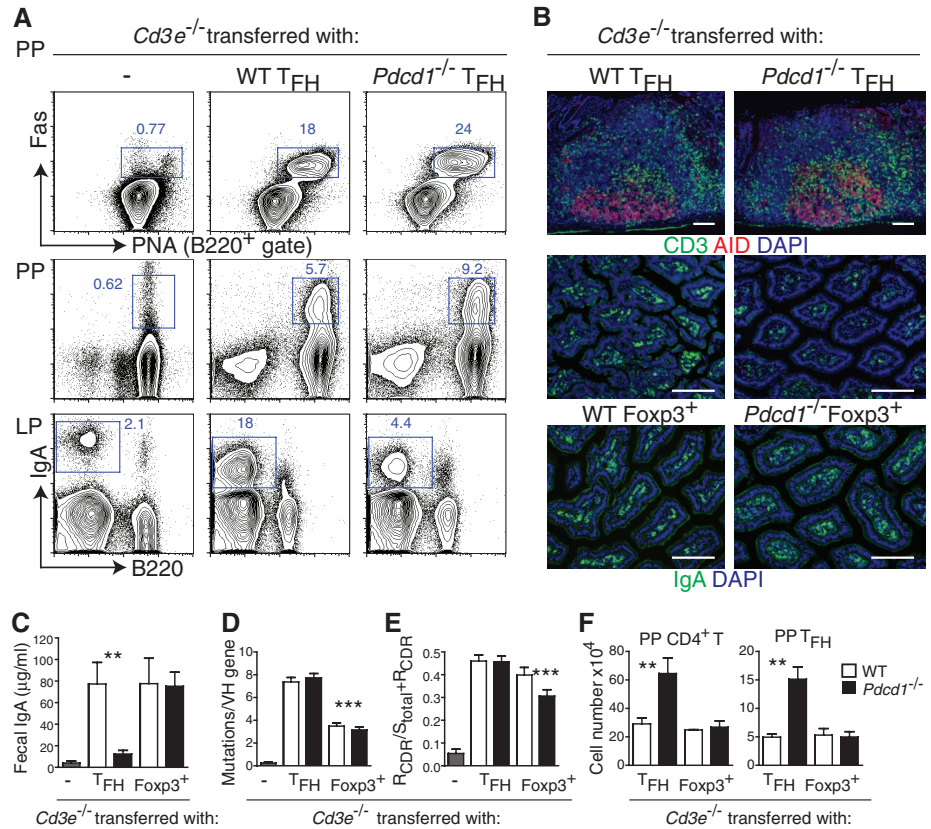


Fig. 4. T_{FH} cells from *Pdcd1*^{-/-} mice are impaired in their ability to support the generation of IgA plasma cells in gut. (A) Representative flow cytometric profiles of cells isolated from PPs and LP stained for the indicated markers. Numbers represent percentage of cells in the indicated gates. (B) Sections of the PPs and small intestine stained as indicated and (C) fecal IgA levels as determined by ELISA from the *Cd3e*^{-/-} mice 3 months after reconstitution with T_{FH} cells and Foxp3⁺ T cells from WT and *Pdcd1*^{-/-} mice. Scale bars, 100 μm. (D) Absolute number of somatic mutations in V_H genes and (E) the affinity maturation of the IgA-producing cells from the LP of *Cd3e*^{-/-} mice at 3 months after the reconstitution with the T cells indicated. The number of sequences analyzed: WT, T_{FH} = 171; *Pdcd1*^{-/-}, T_{FH} = 175; WT Foxp3⁺, T = 153; *Pdcd1*^{-/-} Foxp3⁺, T = 177. (F) Total numbers of indicated cells from the PPs of *Cd3e*^{-/-} mice at 3 months after the reconstitution with the T cells indicated. Means ± SEM (n = 2 to 3 mice per group). Two-tailed unpaired Student's *t* test was used for all statistical analyses; ****P* < 0.001; ***P* < 0.01.

increased turnover (which mirrored the intact *Pdcd1*^{-/-} mice) and a comparable number of mutations in their V_H genes and affinity maturation index with those induced with the help of WT T_{FH} cells (Fig. 4, D and E, and fig. S3A). Similar to what we observed in *Pdcd1*^{-/-} mice, the absolute number of PP T_{FH} cells was much higher in *Cd3e*^{-/-} mice that received *Pdcd1*^{-/-} T_{FH} cells than in mice injected with WT T_{FH} cells, and the *Pdcd1*^{-/-} T_{FH} cells maintained their skewed cytokine profile (Fig. 4F, and fig. S3, B and C). The results indicate that T_{FH} cells in *Pdcd1*^{-/-} mice preclude the generation of IgA-secreting cells in gut. On the other hand, both PD-1-deficient and sufficient Foxp3⁺ T cells, which, as previously reported (22), did not expand much but converted into IL-21-producing T_{FH} cells in PPs, supported the generation of LP IgAs after the transfer into *Cd3e*^{-/-} mice (Fig. 4, B, C, and F; and fig. S3, D and E). It was interesting that the IgAs induced by Foxp3⁺ T cells had significantly fewer mutations and low af-

finity maturation compared with those induced upon transfer of T_{FH} cells (Fig. 4, D and E). These observations suggest that a significant fraction of PCs in *Pdcd1*^{-/-} mice may be generated with the help provided by T_{FH} cells derived from Foxp3⁺ T cells.

Taken together, the results indicate that (i) the quality of gut IgAs critically depends on the number and the nature of T_{FH} cells in PPs; (ii) PD-1 deficiency interferes with the selection of B cells in GCs; and (iii) in addition to selection in PP GCs, IgAs appear to undergo a second, commensal-driven selection in the LP. Thus, the LP may serve not only as reservoir of PC but also as a site where proliferating PB are reselected to fit the geographical distribution of dynamic bacterial communities along the intestine.

In *Pdcd1*^{-/-} mice, as in SHM-defective AID^{G23S} mice (23), the gut microbiota induce hyperactivation of the systemic immune system (fig. S4, A and B). Indeed, along with T and B cell hyperplasia, we found that serum

from *Pdcd1*^{−/−} mice contained antibodies specific for components of commensal bacteria, which indicated a breach of normal mucosal-systemic compartmentalization (fig. S4, A and C) (24). On the basis of the data presented here, we propose that the skewed gut microbial communities that result from the dysregulated selection of IgAs drive the expansion of self-reactive B and T cells. Our studies have implications for how modulation of PD-1 expression promotes tolerance or uncontrolled immune reactions leading to autoimmunity.

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Supplementary Materials

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Materials and Methods
Figs. S1 to S4
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Microbial Exposure During Early Life Has Persistent Effects on Natural Killer T Cell Function

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Exposure to microbes during early childhood is associated with protection from immune-mediated diseases such as inflammatory bowel disease (IBD) and asthma. Here, we show that in germ-free (GF) mice, invariant natural killer T (iNKT) cells accumulate in the colonic lamina propria and lung, resulting in increased morbidity in models of IBD and allergic asthma as compared with that of specific pathogen-free mice. This was associated with increased intestinal and pulmonary expression of the chemokine ligand CXCL16, which was associated with increased mucosal iNKT cells. Colonization of neonatal—but not adult—GF mice with a conventional microbiota protected the animals from mucosal iNKT accumulation and related pathology. These results indicate that age-sensitive contact with commensal microbes is critical for establishing mucosal iNKT cell tolerance to later environmental exposures.

The mammalian host immune system is broadly stimulated with the first exposure to microorganisms during neonatal life (1). The inner surfaces of the gastrointestinal tract and lungs are particularly affected because they are predominant sites of microbial contact (2). Epidemiologic observations further suggest that the immune effects of early-life microbial exposure are durable and persist into later life because they can be associated with prevention of diseases such as inflammatory bowel disease (IBD) and asthma (3–5).

Invariant natural killer T (iNKT) cells probably play an important role in the pathogenesis of ulcerative colitis (UC)—a major form of IBD—and in asthma (6, 7). Such cells recognize endogenous and exogenous lipid antigens presented by the nonpolymorphic major histocompatibility complex (MHC) class I-like protein CD1d and

secrete abundant amounts of proinflammatory cytokines such as interleukin-4 (IL-4) and IL-13 upon activation (8, 9). We therefore investigated age-dependent regulation of iNKT cells by use of microbes in mouse models of IBD and asthma.

We first examined the appearance of iNKT cells in tissues of 8-week-old germ-free (GF) and specific pathogen-free (SPF) Swiss-Webster (SW) mice. Relative and absolute numbers of iNKT cells were increased in GF mice in colonic lamina propria (LP) (Fig. 1, A to C). These differences in colonic iNKT cell numbers between GF and SPF mice were detectable after weaning and stable for life, suggesting early and persistent effects of the microbiota (Fig. 1D). iNKT cells were not increased in the ileal LP (ileum) of GF mice, and the liver, spleen, and thymus contained even fewer iNKT cells under GF relative to SPF conditions (fig. S1), which is consistent with a recent

report (10). GF C57BL/6 mice (B6) exhibited similar increases of iNKT cells in the colonic LP as well as the liver, in contrast to GF SW mice (fig. S2). Although increased in number, the iNKT cell expression of several activation and memory markers was unaltered in GF SW, relative to SPF, mice (fig. S3).

To examine the relevance of these findings, we investigated the susceptibility of GF and SPF mice to oxazolone-induced colitis, a model of intestinal inflammation that possesses features of UC and is dependent on IL-13 production by CD1d-restricted iNKT cells (11, 12). Although GF or germ-reduced mice exhibit exacerbated inflammation in an innate mouse model of colitis (13, 14), colitis is typically prevented under GF conditions in models dependent on an adaptive immune response (15). Surprisingly, GF mice were more sensitive to oxazolone-induced colitis, as revealed by severe weight loss, pathology, and a high mortality rate in contrast to SPF mice (Fig. 1, E to H). This was not due to overexpression of cell-surface CD1d expression on intestinal epithelial (fig. S4A) and hematopoietic cells (fig. S4B). Consistent with previous studies (11), the

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colitis in GF mice was characterized by a marked increase in production of IL-13 and IL-1 β in comparison with SPF mice (Fig. 1I).

Fig. 1. Intestinal bacteria-dependent accumulation of colonic iNKT cells in GF mice leads to high mortality in oxazolone-induced colitis. (A to C) The percentage of CD1d tetramer–positive cells (iNKT cells) within the live lymphocytes from the lamina propria (LP) of gender-matched GF and SPF SW mice (7 to 8 weeks old) was analyzed by means of flow cytometry (A). Representative dot-plots are shown in (B), and the absolute number of iNKT cells is shown in (C). Each circle in (A) represents an individual mouse. (D) Percentages of colonic iNKT cells of live LP lymphocytes in gender-matched SPF and GF mice were analyzed at different ages by means of flow cytometry ($n = 4$ mice per group). (E to H) Eight-week-old GF and SPF mice were monitored and scored after rectal challenge with 1% oxazolone (ox) or 50% ethanol for survival and body weight loss for 5 days. On day 5, the colons were collected and dissected for histological analysis ($n = 5$ mice per group). (G) Scale bar, 50 μ m. (I) The concentration of IL-4, IL-13, and IL-1 β in the supernatant of 24 hours–colon organ explant cultures determined by means of enzyme-linked immunosorbent assay (ELISA) on day five ($n = 5$ mice per group). All data were obtained from three independent experiments with similar results. In all panels, error bars represent the SD. $^*P < 0.05$, $^{**}P < 0.01$, unpaired t test and $^*P < 0.05$, log-rank test in (H).

To confirm the CD1d-restriction of this colitis in GF mice, we investigated the effects of CD1d blockade. Adult (8- to 9-week-old) GF and SPF mice were treated with a blocking monoclonal antibody (Ab) specific for CD1d (19G11) or an isotype control Ab (16). Treatment of GF mice

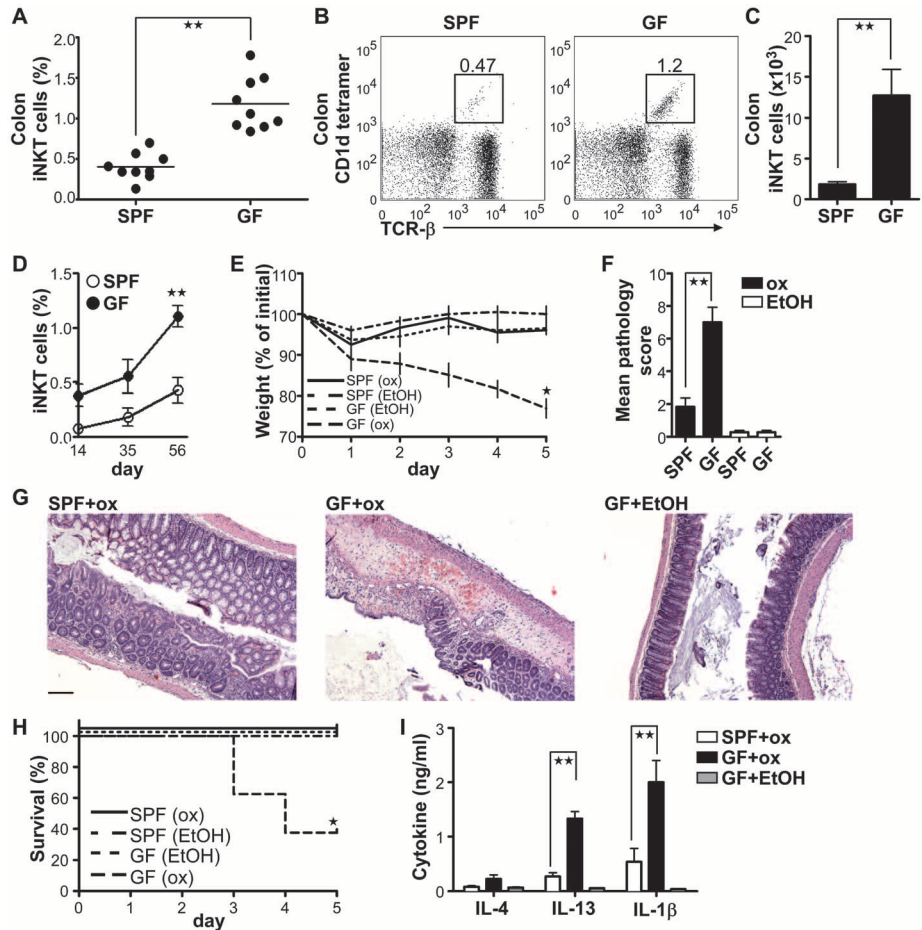
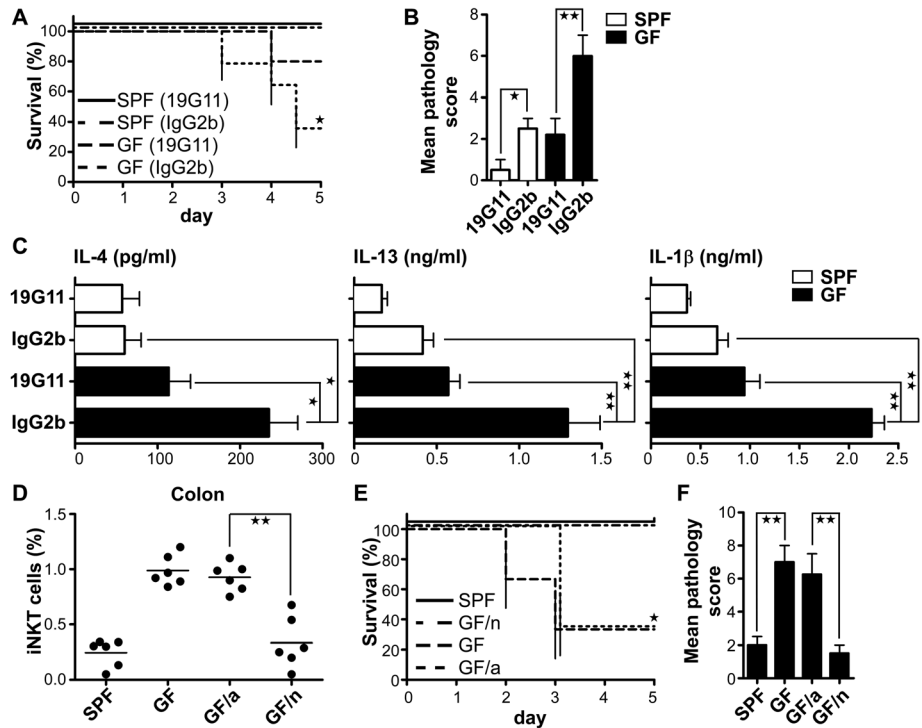


Fig. 2. Microbial colonization during early life prevents the CD1d/iNKT cell-dependent high mortality in oxazolone colitis in GF mice. (A to C) Eight-week-old GF and SPF mice were treated once with 1 mg of 19G11 or isotype control IgG2b antibody before presensitization and rectal oxazolone challenge. (A) Survival after oxazolone challenge is shown. (B) On day 5, the colons were collected for histological analysis, and (C) the cytokine concentration in the supernatant of 24 hours–colon organ explant cultures was measured with ELISA ($n \geq 4$ mice per group). (D) Colonic LP lymphocytes were analyzed for iNKT cells by flow cytometry. (GF/a) mice were GF mice that were exposed to SPF environmental conditions at the age of 5 weeks and maintained for 4 more weeks. (GF/n) mice were pups exposed to SPF conditions on their first day of life and maintained under SPF environmental conditions for 8 to 9 weeks. Each circle represents a mouse. (E to F) Colonized mice were treated as above with oxazolone ($n = 5$ mice per group). All data were obtained from more than two independent experiments with similar results. Error bars indicate SD. $^*P < 0.05$, $^{**}P < 0.01$, unpaired t test and $^*P < 0.05$, log-rank test in (A) and (E).



with 19G11, but not the isotype control, protected against colitis-induced mortality and associated pathology (Fig. 2, A to C, and fig. S4, C and D). Furthermore, CD1d blockade of GF or SPF mice did not lead to significant functional changes in dendritic cells (17) or B cells (18) as demon-

strated by stable IL-12 or IL-10 production (fig. S4, E and F), respectively.

We next examined whether reestablishing microbiota in adult GF mice would normalize iNKT cell levels in the colon. Quite surprisingly, exposure of adult GF mice to SPF conditions (GF/a)

did not restore iNKT cells in the colon to the levels observed in SPF mice or reverse the mortality and severe pathology after oxazolone administration (Fig. 2, D to F). We therefore considered whether the ability to normalize iNKT cell levels and function in the colon was dependent on the

Fig. 3. The increased CD1d/iNKT cell-mediated allergic response sensitivity of GF mice is dependent on age of colonization. (A to C) Lymphocytes from lungs of 8-week-old mice in each group were analyzed for iNKT cells by means of flow cytometry (A). Representative dot-plot is shown in (B), and the absolute number of iNKT cells is shown in (C) ($n = 6$ mice per group). (D to F) Age-matched mice from each group were treated once before OVA presensitization with 1 mg of 19G11 or IgG2b isotype control antibody and with 0.5 mg of 19G11 or IgG2b antibody before the first and the third serial OVA exposure. Twenty-four hours after the last challenge with 5% aerosolized OVA, the mice were analyzed for (D) airway resistance (R_n), (E) percentage of eosinophils of total BALF cells, and (F) IgE ($n = 4$ mice per group). (G) Lung lymphocytes were analyzed for iNKT cells by means of flow cytometry of age- and gender-matched GF/n and GF/a mice. (H to J) Mice were presensitized with OVA and analyzed after the last serial OVA challenge as described above ($n \geq 4$ mice per group). Each circle in (A), (D), (G), and (H) represents an individual mouse. All data were obtained from more than two independent experiments with similar results. Error bars indicate the SD. * $P \leq 0.05$, ** $P < 0.01$, unpaired t test.

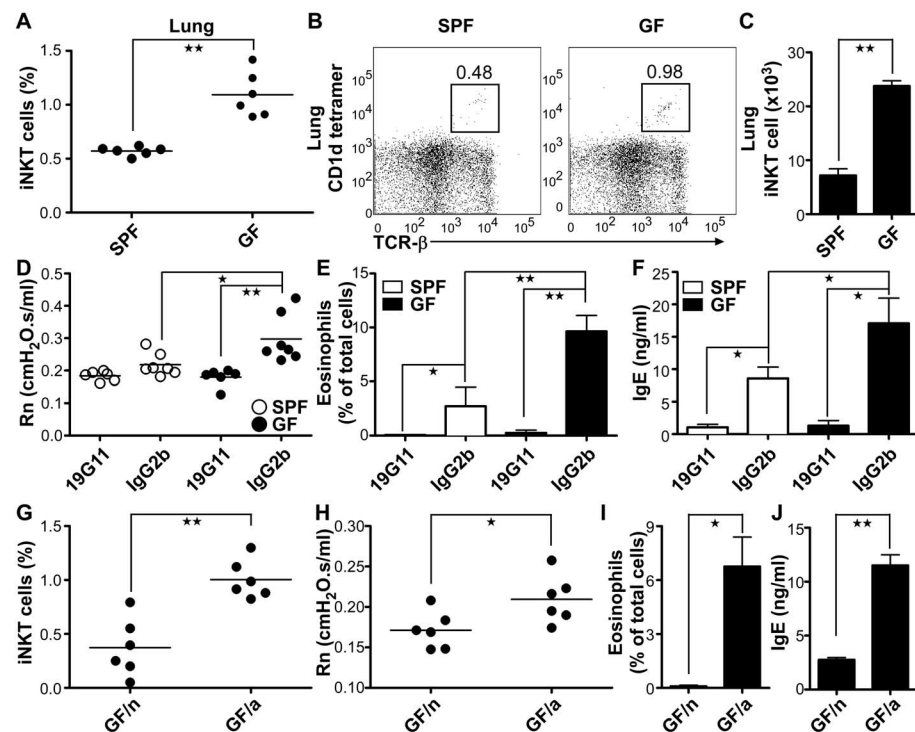
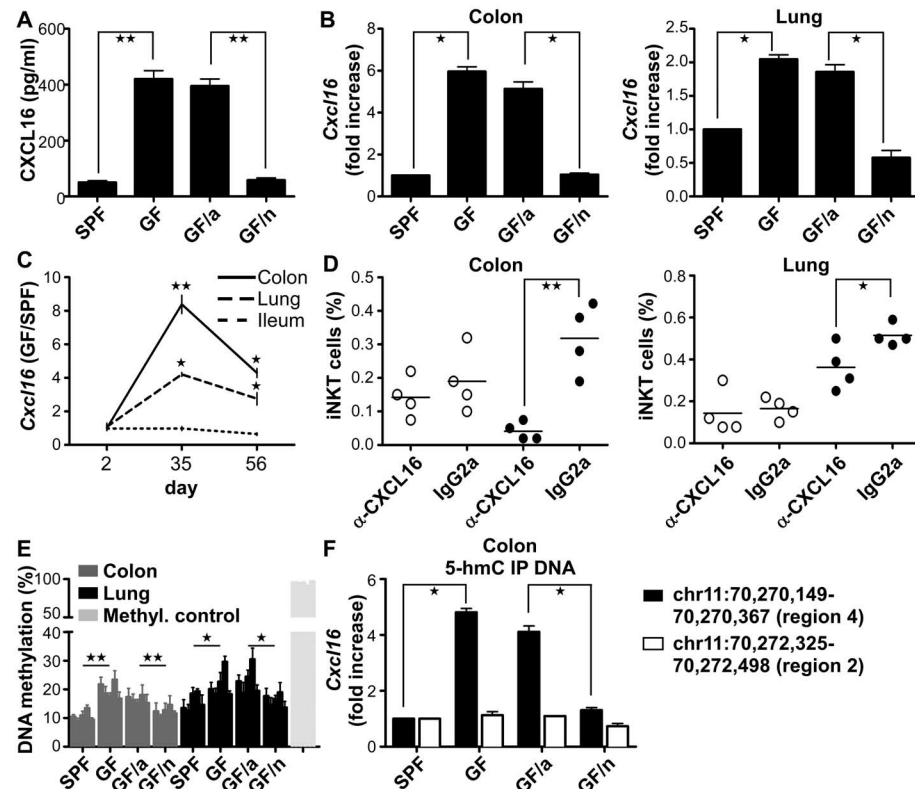


Fig. 4. Microbiota affects tissue specific iNKT cell accumulation by genetic modifications of *Cxcl16*. (A) The CXCL16 concentration in the serum of each group was measured with ELISA in SPF, GF, GF/a, and GF/n as described above ($n = 4$ mice per group). (B) Colon and lung tissue samples were harvested from age-matched mice from each group and analyzed for *Cxcl16* expression ($n = 4$ mice per group). (C) Colon, ileum, and lung tissue samples were analyzed from age-matched SPF and GF mice at different time points for *Cxcl16* expression ($n = 5$ mice per group). (D) SPF (open circles) and GF (closed circles) newborn mice were treated three times a week intraperitoneal with 25 μ g of a neutralizing CXCL16 antibody (α CXCL16) or its isotype control IgG2a antibody and analyzed at the age of 2 weeks for iNKT cell percentages by means of flow cytometry. Each circle represents a mouse. (E) Analysis of DNA methylation of five CpG sites of *Cxcl16* by bisulfite pyrosequencing. For each group, the mean of DNA methylation of 5 CpG sites is shown as a percentage according to the methylated control (Methyl. control) ($n \geq 3$ mice per group). (F) *Cxcl16* qPCR analysis of colonic DNA after performing a 5-hydroxymethylated DNA immunoprecipitation (5-hmC IP DNA) ($n \geq 3$ mice per group). All data were obtained from at least two independent experiments with similar results. Error bars indicate the SD. * $P \leq 0.05$, ** $P < 0.01$, unpaired t test.



age at which microbial contact occurred. Indeed, when we colonized pregnant GF female mice just before delivery and therefore exposed neonatal GF mice to SPF conditions on their first day of life (GF/n), we observed a complete normalization of iNKT cell levels that persisted even two months after colonization (Fig. 2D). Consistent with this, GF/n exhibited reduced susceptibility to oxazolone-induced colitis (relative to that of GF mice) two months after colonization, with a degree of severity identical to that observed in SPF animals, as shown by an analysis of mortality, weight loss, pathology, and cytokine production (Fig. 2, E and F, and fig. S5).

To verify that the early life events associated with the absence of normal microbial colonization on iNKT cell homeostasis were CD1d-dependent, we treated GF mice with 19G11 Ab for their first 6 weeks of life. 19G11, but not control Ab, treatment blocked accumulation of iNKT cells in all tissues examined and susceptibility to oxazolone-induced colitis in later life (fig. S6). Similar to GF mice, SPF mice (B6) born under germ-reduced conditions exhibited an increase in colonic iNKT cells and excessive oxazolone-induced colitis at 4 weeks of life (fig. S7). As predicted (11), *Cd1d*^{-/-} and *Ja18*^{-/-} mice, both of which lack iNKT cells, were not susceptible to oxazolone-induced colitis either under SPF or germ-reduced conditions (fig. S7).

Animal models of asthma demonstrate an important role for iNKT cells (19, 20), although the contribution to human disease remains controversial (21). We found that the lungs of GF SW and B6 mice contained significantly higher relative and absolute numbers of iNKT cells in comparison with that of the lungs of the respective SPF mice (Fig. 3, A to C, and fig. S8). Because commensal microbes may affect the induction of experimental asthma (22), we tested an ovalbumin (OVA)-driven allergic-asthma mouse model in GF mice. We observed that similar to the colon, GF mice developed an allergic airway response to OVA significantly greater than that observed in SPF mice, as demonstrated by increases in airway resistance, total bronchial alveolar lavage fluid (BALF) cell numbers, BALF eosinophilia, serum immunoglobulin E (IgE) levels, proinflammatory cytokine production in the BALF, and lung tissue eosinophil infiltration (Fig. 3, D to F, and fig. S9). The asthma in GF mice was CD1d- and antigen-dependent because elimination of the asthmatic response was only observed with 19G11, but not control, Ab treatment (Fig. 3, D to F, and fig. S9) of OVA-sensitized mice but not mice exposed to phosphate-buffered saline (PBS), which did not induce allergic asthma (fig. S10).

We therefore next investigated whether early-life exposure to a conventional microbiota also protects animals from CD1d-restricted inflammation in the lungs. As observed in the intestinal mucosa, neonatal (GF/n)—but not adult-life (GF/a)—exposure of GF mice to a conventional microbiota abrogated the increased accumulation of iNKT cells in the lungs and protected adult

mice in the allergic asthma model from the pathology (Fig. 3, G to I, and fig. S11).

The chemokine receptor CXCR6 on iNKT cells (23) and its ligand CXCL16, which is expressed at high levels by human epithelial cells (24) and increased in inflammation (25), plays an important role in iNKT cell homeostasis. Therefore, we examined the serum of GF and GF/a mice for the presence of CXCL16 and observed significantly higher levels than that observed in SPF and GF/n mice (Fig. 4A). This was due to significant increases of *Cxcl16* mRNA expression levels in the colon and lung—but not ileum, liver, and thymus—of GF and GF/a relative to SPF and GF/n mice (Fig. 4B and fig. S12A), and mainly immunolocalized to the epithelium (fig. S12B). Although *Cxcl16* mRNA levels in the colon, ileum, and lung were similar immediately after birth in the GF and SPF animals at the beginning of colonization, they increased significantly and specifically only in the colon and lungs of the GF mice during later life (Fig. 4C). These results suggest that microbial exposure provides signals that determine *Cxcl16* gene expression in specific tissues. GF and SPF mice, however, exhibited similar levels of *Cxcr6* mRNA expression in tissues and CXCR6 expression on the cell surface of iNKT cells (fig. S12, C and D).

CXCL16 was responsible for the accumulation of iNKT cells because treatment of newborn SPF and GF mice with a CXCL16-neutralizing Ab caused a decrease in iNKT cells in the colon and lung (Fig. 4D) but had no effect on iNKT cell levels in the ileum or thymus (fig. S13A). However, it was associated with an increased accumulation of iNKT cells in the liver (fig. S13A), suggesting CXCL16 blockade caused a redirection of iNKT cells to this organ. Consequently, Ab-to-CXCL16—but not isotype-treated GF mice were protected from oxazolone colitis-induced mortality and pathology (fig. S13, B to D).

We next examined the epigenetic content of the *Cxcl16* gene of GF and SPF mice. A region 5' of the *Cxcl16* gene of SW mice that contains five potential CpG sites was determined by means of bisulfite pyrosequencing to be hypermethylated in the colon and lungs (Fig. 4E)—but not in other tissues such as the spleen and liver (fig. S14)—under GF conditions as compared with SPF conditions. Colonization of neonatal—but not adult—GF mice with a conventional microbiota decreased the hypermethylation of the *Cxcl16* gene to SPF levels (Fig. 4E). Gene activation due to hypermethylation may occur as a consequence of specific types of environmental exposure (26). To confirm this, we treated SPF neonates on their first day of life by oral gavage with high doses of folic acid as previously described (27) so as to force *Cxcl16* methylation. Compared with PBS-treated control mice, folic acid administration resulted in elevated methylation of the *Cxcl16* gene in the colon, ileum, and lung; at least a three- to fourfold increase of *Cxcl16* mRNA expression in the same tissues; increased CXCL16 serum levels; and accumulation of iNKT cells in

these tissues as observed in GF mice (fig. S15). Alternatively, we investigated whether elimination of *Cxcl16* methylation in GF mice would reverse the elevation of CXCL16 expression by treating GF newborn littermates with the DNA methyltransferase inhibitor 5-Azacytidine (5-Aza). When examined at 2 weeks of life, 5-Aza treatment inhibited methylation and mRNA expression of *Cxcl16* in the colon, ileum, and lung (fig. S16, A and B), diminished the levels of CXCL16 in the serum, and reduced iNKT cells in these tissues (fig. S16, C and D). 5-Aza treatment had no effect on other cell populations such as CD45⁺CD11c⁺ double positive cells (fig. S17).

Epigenetic marks provided by 5-hydroxymethylcytosine (5-hmC) incorporation into DNA have been suggested to be different from those provided by 5-methylcytosine (5-mC) and a distinct signature for elevated levels of transcription (28). We therefore performed DNA immunoprecipitation with a monoclonal 5-hmC Ab followed by quantitative polymerase chain reaction (PCR) analysis for *Cxcl16* in seven different regions of *Cxcl16* in DNA samples obtained from the colons of SPF, GF, GF/a, and GF/n mice. We observed that *Cxcl16* was highly increased in three out of the seven investigated regions (with region 4 as a representative) within the 5-hmC-modified DNA of GF and GF/a mice but not in the DNA acquired from SPF and GF/n mice (Fig. 4F). No differences were observed in the other four regions (with region 2 as a representative) and in the DNA samples obtained from the ileum demonstrating the colonic specificity for 5-hmC modification of *Cxcl16* (fig. S18). A map summarizing this information is provided in fig. S19.

Our studies indicate that CXCL16 is an age- and organ-dependent microbially regulated factor that modulates the quantities and function of iNKT cells in the colon and lungs and, consequently, susceptibility to tissue inflammation. The exact mechanism by which the microbiota regulates CXCL16 expression and thus iNKT cell accumulation in these organs is unknown, although it is independent of the Toll-like receptor adapter protein MyD88 (fig. S20). These observations are in accordance with previous epidemiological studies collectively known as the “hygiene hypothesis,” which proposes that early-life exposure to specific microbe-enriched environments decreases susceptibility to diseases such as IBD and asthma (3, 5, 29, 30), whereas its absence, as in antibiotic treatment during childhood, may have the opposite effect (31, 32). Our results suggest that CD1d-restricted iNKT cell pathways and their relationship to microbes play a central role in these aforementioned processes. Early-life microbial exposure elicits long-lasting effects on iNKT cells, and in their absence, later-life exposure to factors that stimulate these cells may induce an autoinflammatory response. These findings are predicted to extrapolate to humans, given the extensive similarities between the mouse and human CD1d and iNKT cell systems (33).

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Supplementary Materials

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Materials and Methods

Figs. S1 to S20

Tables S1 and S2

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Analytic Thinking Promotes Religious Disbelief

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Scientific interest in the cognitive underpinnings of religious belief has grown in recent years. However, to date, little experimental research has focused on the cognitive processes that may promote religious disbelief. The present studies apply a dual-process model of cognitive processing to this problem, testing the hypothesis that analytic processing promotes religious disbelief. Individual differences in the tendency to analytically override initially flawed intuitions in reasoning were associated with increased religious disbelief. Four additional experiments provided evidence of causation, as subtle manipulations known to trigger analytic processing also encouraged religious disbelief. Combined, these studies indicate that analytic processing is one factor (presumably among several) that promotes religious disbelief. Although these findings do not speak directly to conversations about the inherent rationality, value, or truth of religious beliefs, they illuminate one cognitive factor that may influence such discussions.

Although most people fervently believe in God or gods, there are nonetheless hundreds of millions of nonbelievers worldwide (1), and belief and disbelief fluctuate across situations and over time (2). Religious belief and disbelief are likely complex, multi-determined, psychologically and culturally shaped phenomena, yet to date little experimental research has explored the specific cognitive underpinnings of religious disbelief (3, 4). Here we begin to address this important gap in the literature by applying a dual-process cognitive framework, which predicts that analytic thinking strategies might be one potent source of religious disbelief.

According to dual-process theories of human thinking (5, 6), there are two distinct but interacting systems for information processing. One

(System 1) relies upon frugal heuristics yielding intuitive responses, while the other (System 2) relies upon deliberative analytic processing. Although both systems can at times run in parallel (7), System 2 often overrides the input of system 1 when analytic tendencies are activated and cognitive resources are available. Dual-process theories have been successfully applied to diverse domains and phenomena across a wide range of fields (5, 6, 8, 9).

Available evidence and theory suggest that a converging suite of intuitive cognitive processes facilitate and support belief in supernatural agents, which is a central aspect of religious beliefs worldwide (10–13). These processes include intuitions about teleology (14), mind-body dualism (13), psychological immortality (15), and mind perception (16, 17). Religious belief therefore bears many hallmarks of System 1 processing.

If religious belief emerges through a converging set of intuitive processes, and analytic processing can inhibit or override intuitive processing,

then analytic thinking may undermine intuitive support for religious belief. Thus, a dual-process account predicts that analytic thinking may be one source of religious disbelief. Recent evidence is consistent with this hypothesis (4), finding that individual differences in reliance on intuitive thinking predict greater belief in God, even after controlling for relevant socio-demographic variables. However, evidence for causality remains rare (4). Here we report five studies that present empirical tests of this hypothesis.

We adopted three complementary strategies to test for robustness and generality. First, study 1 tested whether individual differences in the tendency to engage analytic thinking are associated with reduced religious belief. Second, studies 2 to 5 established causation by testing whether various experimental manipulations of analytic processing, induced subtly and implicitly, encourage religious disbelief. These manipulations of analytic processing included visual priming, implicit priming, and cognitive disfluency (18, 19). Third, across studies, we assessed religious belief using diverse measures that focused primarily on belief in and commitment to religiously endorsed supernatural agents. Samples consisted of participants from diverse cultural and religious backgrounds (20).

Study 1 was a correlational study with Canadian undergraduates ($N = 179$). We correlated performance on an analytic thinking task with three related, but distinct, measures of religious belief. The analytic thinking task (6) contains three problems that require participants to analytically override an initial intuition. This task was designed to specifically measure analytic processing because an intuitive reading of each problem invites a quick and easy, yet incorrect, response that must be analytically overridden (Table 1). Furthermore, experimental manipulations known to induce analytic processing

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Table 1. Summary of measures used. Asterisks (*) denote reverse-scored items.

Study 1. Analytic thinking task (5)	Intuitive answer	Analytic answer
A bat and a ball cost \$1.10 in total. The bat costs \$1.00 more than the ball. How much does the ball cost? ____cents	10	5
If it takes 5 machines 5 min to make 5 widgets, how long would it take 100 machines to make 100 widgets? ____minutes	100	5
In a lake, there is a patch of lily pads. Every day, the patch doubles in size. If it takes 48 days for the patch to cover the entire lake, how long would it take for the patch to cover half of the lake? ____days	24	47

Intrinsic religiosity (21), study 1 $\alpha = 0.90$
My faith involves all of my life.
I try hard to carry my religion over into all my other dealings in life.
In my life I feel the presence of the Divine.
Nothing is as important to me as serving God as best I know how.
My faith sometimes restricts my actions.
One should seek God’s guidance when making every important decision.
My religious beliefs are what really lie behind my whole approach to life.
*It doesn’t matter so much what I believe as long as I lead a moral life.
*Although I am a religious person, I refuse to let religious considerations influence my everyday affairs.
*Although I believe in my religion, I feel there are many more important things in life.

Intuitive religious belief, study 1 $\alpha = 0.80$
I believe in God
When I am in trouble, I find myself wanting to ask God for help
* When people pray they are only talking to themselves
* I just don’t understand religion
* I don’t really spend much time thinking about my religious beliefs

Belief in supernatural agents, study 1 $\alpha = 0.91$
God exists
The devil exists
Angels exist

reliably improve performance on the task (18). After completing the analytic thinking task, participants completed three different measures of religious belief, including a widely used 10-item intrinsic religiosity scale (Religiosity) (21), a new five-item intuitive religious belief scale (Intuitive), and another scale assessing belief in religious supernatural agents (Agents: God, angels, the devil). Table 1 presents all items from all measures. The three religious belief scales were all highly interrelated, providing evidence for convergent validity; all correlation coefficients (r ’s) were between 0.77 and 0.80, and all P values (P ’s) were <0.001.

In study 1, as hypothesized, analytic thinking was significantly negatively associated with all three measures of religious belief, $r_{\text{Religiosity}} = -0.22, P = 0.003$; $r_{\text{Intuitive}} = -0.15, P = 0.04$; and $r_{\text{Agents}} = -0.18, P = 0.02$. This result demonstrated that, at the level of individual differences, the tendency to analytically override intuitions in reasoning was associated with religious disbelief, supporting previous findings (4).

Studies 2 to 5 tested causation by using experimental manipulations to elicit analytic thinking. We took considerable steps to remove potential effects of experimental demand. In all experiments, instructions were fully automated. In addition, all experimental manipulations used subtle techniques to elicit analytic thinking. Across studies, funnel debriefings revealed that partic-

ipants only very rarely detected a connection between manipulations and religious belief measures (20).

Study 2 used a visual priming paradigm in which a sample of Canadian undergraduates rated their belief in God (from 0 to 100) after being randomly assigned to view four images (samples provided in Fig. 1) of either artwork depicting a reflective thinking pose (Rodin’s

The Thinker; $N = 26$) or control artwork matched for surface characteristics like color and posture (*Discobolus* of Myron; $N = 31$). A pilot test with different participants ($N = 40$) revealed that this novel priming procedure significantly improved performance on a syllogistic reasoning task that measures analytic tendencies (20). In the present study, as hypothesized, viewing *The Thinker* significantly promoted religious

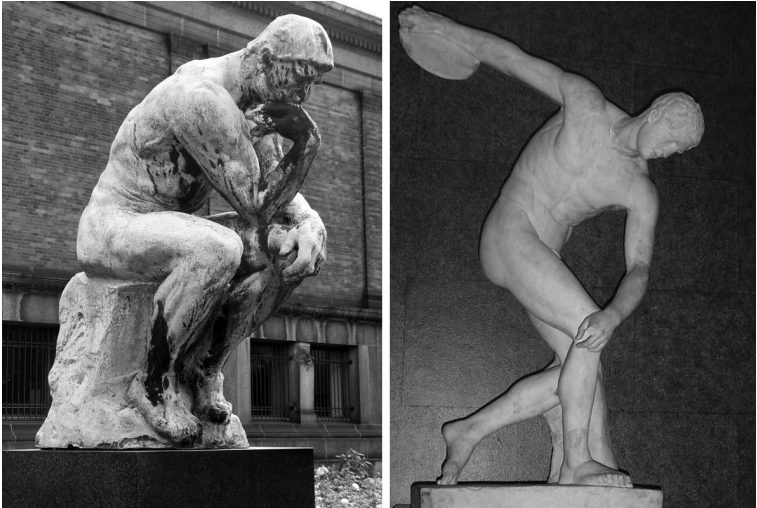


Fig. 1. Sample images of *The Thinker* (left) and *Discobolus* (right) used in study 2. The images shown here are similar to, but not the exact same ones used in the study. [Source: Wikimedia]

Table 2. Summary of experimental methods and findings (studies 2 to 5). *d* reflects effect size estimates (Cohen's *d*).

Study Belief measure (possible range)	Condition: sample stimuli	<i>N</i>	<i>M</i>	<i>SD</i>	<i>t</i>	<i>P</i>	<i>d</i>
2: Art Belief in God (0–100)	Control: <i>Discobolus</i> Analytic: <i>The Thinker</i>	31 26	61.55 41.42	35.68 31.47	2.24	0.03	0.59
3: Implicit Supernatural agents (3–21)	Control: hammer, shoes, jump, retrace, brown Analytic: think, reason, analyze, ponder, rational	43 50	12.65 10.12	5.29 6.13	2.11	0.04	0.44
4: Implicit Intrinsic religiosity (10–70)	Control: hammer, shoes, jump, retrace, brown Analytic: think, reason, analyze, ponder, rational	75 70	40.16 34.39	16.73 14.77	2.20	0.03	0.36
5: Disfluency Supernatural agents (3–21)	Control: sample font Analytic: <i>sample font</i>	88 91	12.16 10.40	5.99 5.44	2.06	0.04	0.31

disbelief [$t(55) = 2.24$, $P = 0.03$, Cohen's $d = 0.60$; Table 2]. In sum, a novel visual prime that triggers analytic thinking also encouraged disbelief in God. Although participants showed no awareness of the hypothesis or the influence of the primes, study 2 nonetheless relied on a fairly overt task to induce analytic processing. To further reduce potential experimental demand, studies 3 to 5 relied on even subtler manipulations to trigger analytic thinking outside of participants' explicit awareness.

In studies 3 and 4, participants rated their religious belief after completing a modified verbal fluency task priming procedure (22) previously used to activate analytic thinking without explicit awareness (23). In this task, participants received 10 different sets of five randomly arranged words (e.g., "high winds the flies plane"). For each set of five words, participants dropped one word and rearranged the others to form a meaningful phrase (e.g., "the plane flies high"). The analytic condition included five-word sets containing target analytic thinking words (analyze, reason, ponder, think, rational), and the control condition included thematically unrelated words (e.g., hammer, shoes, jump, retrace, brown, etc.). Because these exact words have not been used in previous research relating implicit primes to analytic thinking, we performed a pilot test with another group of participants ($N = 79$), which indicated that the analytic thinking primes did, as expected, improve performance on a subsequent analytic thinking task (20).

Study 3 included a sample of Canadian undergraduates who were randomly assigned to either the Analytic ($N = 50$) or the Control ($N = 43$) prime before completing the belief in supernatural agents measure used in study 1 (Table 1). As hypothesized, implicitly primed analytic thinking concepts significantly increased religious disbelief [$t(91) = 2.11$, $P = 0.04$, Cohen's $d = 0.44$; Table 2]. In addition, we obtained a measure of pre-experiment religious belief several weeks before the experimental session

to test whether pre-experiment individual differences in religious belief moderated any effects of analytic thinking on religious belief. Premeasured religious belief did not significantly moderate the effects of the analytic thinking prime on religious belief ($F = 0.42$, $P = 0.66$) (20).

Study 4 replicated the main result of study 3 with a broad nationwide (though nonrepresentative) sample of American adults recruited online, reflecting a wide range in age, income, and education (20, 24). Participants were again randomly assigned to complete either the Analytic ($N = 71$) or the Control ($N = 77$) implicit prime before completing the intrinsic religiosity measure used in study 1 (Table 1). Implicitly primed analytic thinking concepts again increased religious disbelief [$t(143) = 2.20$, $P = 0.03$, Cohen's $d = 0.36$; Table 2]. Combined, studies 3 and 4 demonstrated that even implicitly primed analytic thinking promotes religious disbelief. Nonetheless, experimental manipulations in studies 2 to 4 elicited analytic thinking by having participants perform one task or another (looking at pictures or unscrambling sentences) before rating their religious beliefs. Although unlikely, it is conceivable that the act of performing any task—not just tasks known to elicit analytic cognitive tendencies—may decrease religious belief.

In study 5, we used a still more subtle experimental manipulation that did not even require participants to perform an initial task to activate analytic thinking. We relied on cognitive disfluency, which is known to trigger analytic thinking strategies (18, 19). For example, in previous research, merely presenting information in a difficult-to-read font improves performance on multiple standard tasks used to evaluate analytic thinking in dual-process research, including syllogistic reasoning and the analytic thinking task used in study 1 (18, 19). We capitalized on these established findings by having participants rate their religious beliefs on a questionnaire presented in fonts pre-rated by a separate

group of participants (20) as either typical ($N = 91$; **sample**) or difficult-to-read ($N = 91$; *sample*). As hypothesized, analytic thinking activated via disfluency significantly increased religious disbelief [$t(177) = 2.06$, $P = 0.04$, Cohen's $d = 0.31$; Table 2]. As in study 4, individual differences in pre-experiment religious belief did not moderate the effect of analytic thinking on religious belief ($F < 0.05$, $P = 0.96$) (20). Additional alternative explanations focusing on experimental artifacts introduced by the disfluent font did not receive empirical support (20).

All of the manipulations used in studies 2 to 5 plausibly produce multiple effects, and any specific finding in a given study may be open to alternative explanations and should be interpreted with caution. However, across all studies, it is difficult to think of a broad alternative explanation that could parsimoniously explain why analytically overriding intuitive answers, visual exposure to a thinking pose, implicit priming of analytic thinking concepts, and perceptual disfluency all converge on promoting religious disbelief. By contrast, the hypothesis that analytic processing—which empirically underlies all experimental manipulations—promotes religious disbelief explains all of these findings in a single framework that is well supported by existing theory regarding the cognitive foundations of religious belief and disbelief.

These findings provoke the question of exactly at which stage of processing analytic strategies influence religious belief. We suggest three possibilities for future research. First, analytic processing may directly inhibit the low-level intuitions that presumably support religious beliefs, rather than acting specifically on higher-order religious cognitions. In support of this possibility, manipulations known to interfere with analytic thinking also increase the tendency to engage in teleological thinking (25). Second, engagement with analytic thinking may leave such low-level intuitions operational, yet inhibit the development of higher-order religious beliefs as they begin to arise in appropriate cultural contexts.

That is, people may still draw, for example, on teleological or dualistic intuitions, yet analytically override theistic beliefs. Third, rather than inhibiting low-level intuitions directly, or inhibiting theistic tendencies resulting from intuitive processes, analytic thinking might allow people to reflectively override existing religious beliefs. All three of these possibilities are broadly consistent with the present results, and may be complementary accounts rather than alternatives. We leave these intriguing possibilities for future research.

In closing, we urge caution in interpreting three key implications of the present results. First, although these findings were robust to variation in ethnic and religious backgrounds in the current samples, and in study 4, to variation in other demographic characteristics (20), it is important to examine the generalizability of our findings further across a more diverse range of populations and cultural contexts in future research (26). Second, although these results indicate that analytic processing promotes religious disbelief, we again emphasize that analytic processing is almost certainly not the sole cause of religious disbelief. Disbelief likely also emerges from selective deficits in the intuitive cognitive processes that enable the mental representation of religious concepts such as supernatural agent beliefs (10, 11, 13, 27), from secular cultural contexts lacking cues that one should adopt specific religious beliefs (1, 28, 29), and in societies that effectively guarantee the existential security of their citizens (30). The present results suggest one possible cognitive source of religious disbelief, and join a growing literature using experimental techniques to test hypotheses regarding

the cognitive, motivational, and cultural origins of religious beliefs (31). Finally, we caution that the present studies are silent on long-standing debates about the intrinsic value or rationality of religious beliefs (32, 33), or about the relative merits of analytic and intuitive thinking in promoting optimal decision making (34). Instead, these results illuminate, through empirical research, one cognitive stage on which such debates are played (35).

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Supplementary Materials

www.sciencemag.org/cgi/content/full/336/6080/493/DC1
Materials and Methods
Supplementary Text
Table S1
References (35–37)

24 October 2011; accepted 21 March 2012
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IL-1 β	15.6-500 pg/ml
IL-2	31.2-1000 pg/ml
IL-2 high sensitivity	1.87-60 pg/ml
IL-4	1.1-35 pg/ml
IL-4 high sensitivity	0.31-10 pg/ml
IL-5	7.8-250 pg/ml
IL-6	6.25-200 pg/ml
IL-6 high sensitivity	1.56-50 pg/ml
IL-7	6.25-200 pg/ml
IL-8	62.5-2000 pg/ml
IL-10	12.5-400 pg/ml
IL-10 high sensitivity	1.56-50 pg/ml
IL-12/p70	6.25-200 pg/ml
IL-12 high sensitivity	0.78-25 pg/ml
IL-12/p40 + p70	62.5-2000 pg/ml
IL-13	3.12-100 pg/ml
IL-17A	3.12-100 pg/ml
IL-17F	15.6-500 pg/ml
IL-23	78-5000 pg/ml
IL-27	31.25-1000 pg/ml
IP-10	6.25-200 pg/ml
MCP-1	16-1000 pg/ml
TGF- β 1	0.5-30 ng/ml
TGF- β 2	31.25-1000 pg/ml
TNF- α	25-800 pg/ml
VEGF-A Biolisa	16-1000 pg/ml
VEGF-A	31.25-2000 pg/ml
VEGF-R1	0.16-10 ng/ml

Human Cytokine

Receptors

Range

CD25/IL-2R	68.75-2220 pg/ml
CD116/GM-CSF R	31.25-1000 pg/ml
CD117/c-KIT	0.31-10 ng/ml
CD124/IL-4R	31.25-1000 pg/ml
CD126/gp80	31.25-1000 pg/ml
CD130/gp130	56.25-1800 pg/ml
CD213a2/IL-13R α 2	156-5000 pg/ml
CD138/SYNDECAN-1	8-256 ng/ml

Mouse Cytokines

Range

GM-CSF	15.6-500 pg/ml
IFN- γ	31.25-1000 pg/ml
IL-1 α	7.8-500 pg/ml
IL-2	15.6-500 pg/ml
IL-4	3.9-250 pg/ml
IL-5	7.8-500 pg/ml
IL-6	15.6-500 pg/ml
IL-10	31.25-1000 pg/ml
IL-12	31.25-2000 pg/ml
TGF- β 1	0.5-30 ng/ml
TNF- α	31.25-1000 pg/ml

Rat Cytokines

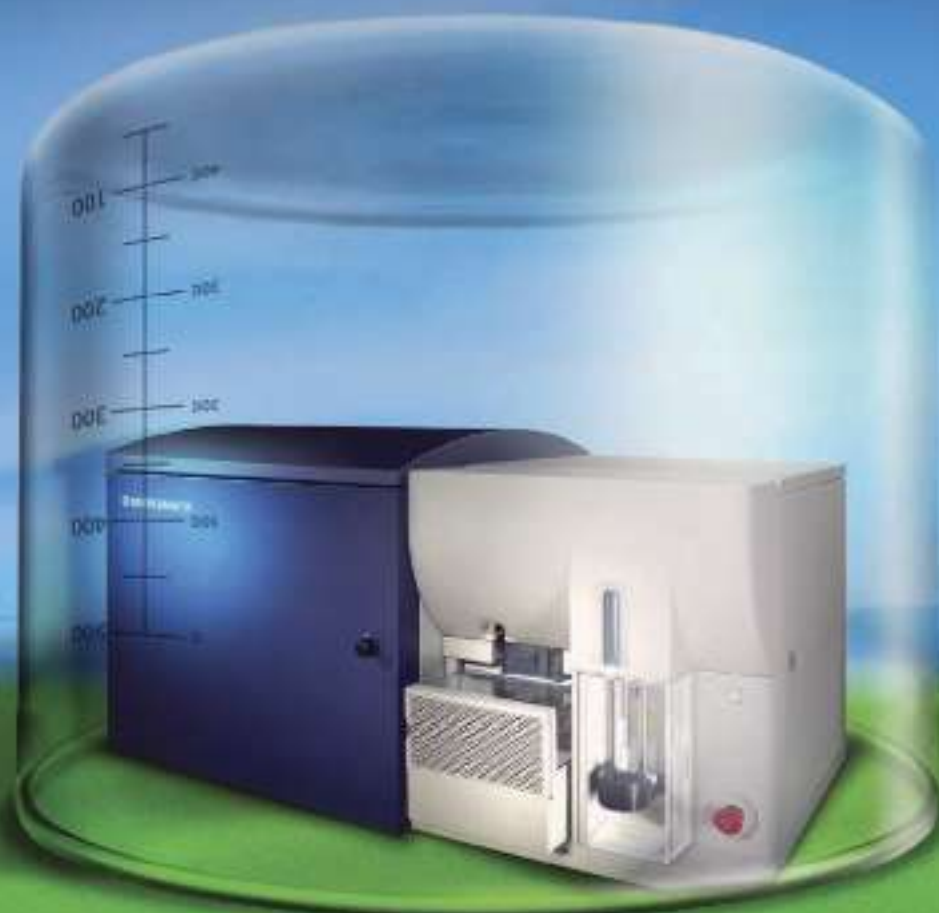
Range

IFN- γ	31.25-1000 pg/ml
IL-1 β	31.25-2000 pg/ml
IL-4	31.25-100 pg/ml
IL-6	31.25-2000 pg/ml
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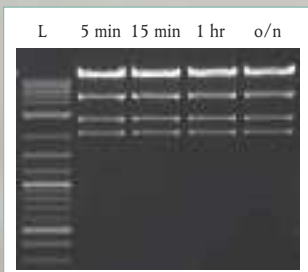
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ARTICLE: *An Electronic Second Skin*
DATE: Sep 21, 7:43am

LOCATION: University Faculty Lounge
ARTICLE: *The Visual Impact of Gossip*
DATE: Sep 21, 4:22pm

LOCATION: Gyro King
ARTICLE: *Cavemen Craved Carbs, Too*
DATE: Sep 21, 1:13pm

LOCATION: Hemlock Bar
ARTICLE: *Quantum Simulation of Frustrated Classical Magnetism in Triangular Optical Lattices*
DATE: Sep 21, 9:21pm

LOCATION: Bed
ARTICLE: *Consciousness: What, How and Why*
DATE: Sep 21, 10:56pm



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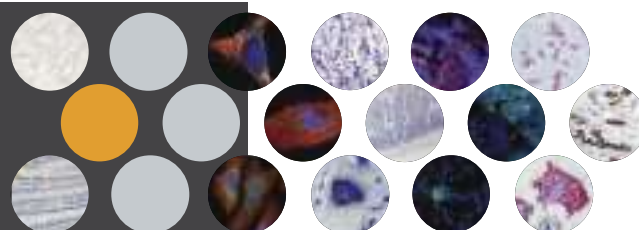
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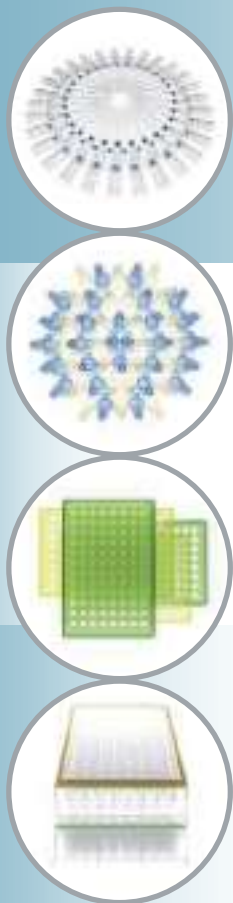
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Phenom-World**For info: +31-402-597360 | www.phenom-world.com****CRYOGENIC STORAGE RACK**

The durably constructed, thick-walled Arctic Rack-24 is a new rack and cover that enables high integrity sample storage at ultralow (cryogenic) temperatures over extended periods of time. The Arctic Rack-24 is designed to accommodate 24 high-volume (6.00 mL or 7.50 mL) Micronic storage tubes in an automation-friendly SBS footprint. A special cover enables the Arctic Rack-24 to be securely locked ensuring high sample security during transport or storage. The Arctic Rack-24 may be autoclaved several times providing considerable cost savings over consumable sample storage products. Absolute traceability and reproducibility on the Arctic Rack-24 are ensured through alphanumeric visual location aids and laser engraved barcodes on two sides of the rack plus rack foot mounting points. The open bottom design of the Arctic Rack-24 facilitates quick defrosting of samples. The Arctic Rack-24 with covers are stackable, enabling conservation of valuable storage space within freezers or during transport.

Micronic**For info: +31-320-277070 | www.micronic.com****REACTION MONITORING SYSTEM**

The new MB-Rx in situ Reaction Monitor is designed for research laboratories and pilot plants in the chemical, petrochemical, pharmaceutical, and biopharmaceutical industries. The MB-Rx is a plug-and-play solution designed around the key concepts of analytical performance, reliability, and simplicity. This robust analyzer provides real-time insight into chemical reaction dynamics. Key parameters such as kinetics over different phases, reagent consumption, and the synthesis of products and by-products can be assessed in real time. The MB-Rx features a rugged Hastelloy ATR probe and an intuitive software interface that also allows for rapid setup of experiment templates. Permanently aligned optics and a light source with an average lifespan of 10 years make the MB-Rx virtually maintenance-free. In addition, it does not use hygroscopic optics or a cryogenic detector, thus eliminating the need for optical purging, desiccant cartridges, liquid nitrogen, or Stirling coolers.

ABB**For info: 800-435-7365 | www.abb.com/analytical****ELECTRONIC PIPETTES**

The new Acura electro Pipettes are ergonomically engineered electronic pipettes designed to provide researchers with the highest level of comfort and precision. The Acura electro Pipette product line consists of three models for an extended range of sample handling performance. The Acura electro 926X model comprises seven micropipettes, ranging in volumes from 0.1 to 1,000 µL; the 936 models includes three macropipettes with volumes ranging from 0.1 to 10 mL; and, the Acura electro 956 offers eight multichannel pipettes with dispensing volumes from 0.5 to 350 µL in 8- or 12-channel configurations. A unique feature of the electro line is that one control unit is capable of fitting on to any one of 27 volumetric assemblies, making electronic pipetting more affordable. Each microprocessor-controlled pipette features a lightweight design for maximum hand comfort and control.

Wheaton**For info: 800-225-1437 | www.wheaton.com****3-D HIGH-CONTENT IMAGING PLATFORM**

Designed for demanding high content assays and cell biology challenges, the new ArrayScan Infinity HCS Reader has 3-D imaging capability and features the latest in variable pin-hole confocal technology, solid-state LED illumination, live-cell and label-free capabilities, coupled with best-in-class image analysis and bioinformatics software. The instrument's integrated confocal module features the latest high-speed Nipkow spinning disk technology and a variable pinhole to give finer control over image acquisition across a range of objectives (magnifications), compared to traditional confocal (fixed pin-hole) technology. The confocal module has a four-color LED Light Engine, bringing robust, laser-like performance without the complications and cost of lasers or the need for alignment of camera, confocal, and laser. The ArrayScan Infinity HCS Reader is also coupled with enhanced Thermo Scientific iDev Workflow software for multidimensional analysis. Multiple projection options for image analysis and the ability to save the stack of images to create 3-D movies enable complex 3-D structures to be imaged more clearly.

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WEBINAR

Next Generation Sequencing and Translational Research

The Express Lane from Bench to Bedside

May 9, 2012

12 noon ET, 9 a.m. PT, 4 p.m. GMT, 5 p.m. UK

Translational research describes the process of translating scientific discoveries into practical (clinical) applications. Genetic profiling to determine more effective, personalized treatments for a variety of diseases is the new reality. Today, top researchers and medical centers are taking next generation sequencing (NGS) data and translating this into improved patient care. In this webinar, you will hear from leading physicians and researchers, and discover how their institutions are taking NGS technology from bench to bedside.

During the webinar, viewers will:

- Learn how the latest NGS technologies are being applied in a clinical setting
- Hear what challenges have been encountered by researchers and how these have been overcome
- Gain insight into what the future might hold for NGS and its role in personalized medicine
- Be able to put questions to the panel in real time!

SPEAKERS

Stephen C. Peiper, M.D.
Thomas Jefferson University
Philadelphia, PA

Steven M. Wolinsky, M.D.
Northwestern University
Chicago, IL

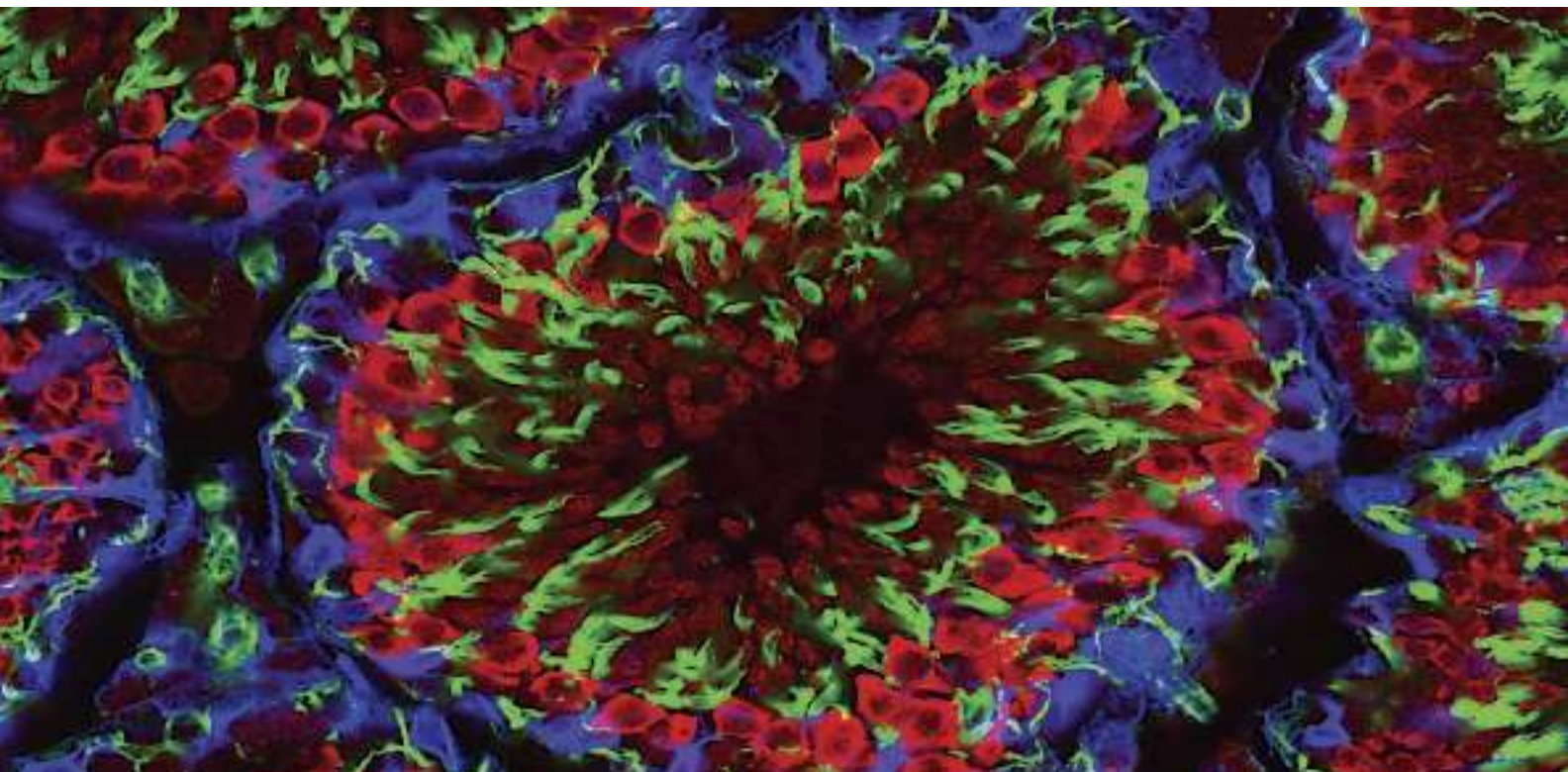
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Science Careers

From the journal *Science*



3 Senior Faculty Positions Cryo Electron Microscopy, X-ray Crystallography, and NMR Sealy Center for Structural Biology and Molecular Biophysics

UTMB Health seeks senior faculty applicants in structural biology in the Sealy Center for Structural Biology and Molecular Biophysics (SCSBMB). The Center supports a graduate program in molecular biophysics and five well-run core laboratories in Cryo-EM, NMR, X-ray, Computation and Solution Biophysics, each with an excellent PhD-level manager. For details see: <http://www.scsb.utmb.edu/>

The successful candidate must be a highly motivated individual with a PhD or MD degree, a strong publication record, and a record of independent well-funded grant support. The ideal candidate will have extensive experience in their specialty applied to the study of the structure, novel mechanisms, and functions of bio-macromolecules. Candidates for positions in either the department of Biochemistry and Molecular Biology or Pharmacology and Toxicology should also have or seek overlap with the highly collaborative established biomedical research community in UTMB's basic science departments, centers and programs of excellence such as the Institute for Human Infection and Immunity, the Galveston National Laboratory, the Center for Tropical Diseases, the Institute for Translational Sciences, the Sealy Center for Cancer Cell Biology, the Sealy Center for Environmental Health and Medicine, the Sealy Center on Aging, the George P. and Cynthia Woods Mitchell Center for Neurodegenerative Diseases, the Moody Center for Brain and Spinal Cord Injury Research, the Sealy Center for Molecular Medicine and the Chemical Biology Program. These and other entities provide a wide variety of core services, in recombinant DNA, genomics, proteomics, high-throughput drug screening, mass spectrometry, membrane protein crystallization, and protein expression and purification. Excellent collaborative opportunities also exist through UTMB's participation in the Gulf Coast Consortia and the Keck Center for Interdisciplinary Bioscience, <http://www.gulfcoastconsortia.org/home.aspx>.

Applicants are requested to submit electronically: a cover letter expressing interest in being considered, a curriculum vitae, current funding, a summary of research accomplishments, and future goals to mail to: SCSBMB.recruiting@UTMB.edu. Direct inquiries to **Dr. B. Montgomery Pettitt**, mpettitt@utmb.edu, 409-772-0723.

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Faculty Positions at Tianjin University: Tianjin, China

Founded in 1895 with the name Peiyang University, Tianjin University is the oldest university in China. As the pioneer of modern higher education in China, the university proudly upholds the motto "Seeking Truth from Facts". Supported by Chinese Ministry of Education, Tianjin University is among the first group of universities to be included in the "985" and "211" Projects of national investment for developing world class universities. Over the years, Tianjin University has grown into a world recognized research university with distinctive quality and strength in education, research and social services. (www.tju.edu.cn)

Academic Disciplines

Tianjin University has a broad range of academic disciplines, covering engineering, natural science, management and some newly emerging inter-disciplines. All disciplines are open to applicants. The university especially encourages research that requires a multi-disciplinary and non-traditional approach. Successful applicants are expected to lead or establish new research directions at Tianjin University.

Positions

Tianjin University invites applications and nominations from outstanding scholars of all nationalities for professorship with full-time arrangements.

Qualifications

Academic Faculties and postdoctoral fellows with outstanding academic performance will be deemed competitive applicants. Relevant research field with a proven record of research excellence and outstanding communication skills are highly essential.

Responsibilities

Responsibilities include teaching undergraduate and graduate courses, developing and sustaining externally funded research programs, supervising graduate student, and professional/institutional services.

Salary and Support

Tianjin University offers competitive salaries, with an annual pre-tax salary of RMB 400K-600K and start-up package that includes adequate start-up financial support, ample laboratory space and research assistants.

Application Procedure

Please refer to <http://hr.tju.edu.cn/new/rsc/zpxx/js/> for detailed application procedure. The application deadline is **30th June 2012**.

Contact Method

Contact Person: Ling Zhang, Qing Du, Section of Recruitment, Human Resource Department, Tianjin University, China. Tel: (+86-022-27403932, 27404886, Fax: (+86-022-27404177, Add: 223/Building 9, 92 Weijin Road, Nankai District, Tianjin, 300072

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This is an outstanding opportunity to lead research that links with ICAS's established strengths in process studies, in order to address important issues such as climate change at regional scales. You will also have the opportunity to contribute to the integration of climate research across the University of Leeds.

For more information about our current climate and atmospheric science research and groups within the School visit www.see.leeds.ac.uk/research/icas/ or the School's website at: www.see.leeds.ac.uk/

The School of Earth and Environment is investing in a number of Chair appointments. Preliminary enquiries about the post may be made to: **Professor Ken Carslaw** on k.s.carslaw@leeds.ac.uk or **0113 343 1597** or **Professor Piers Forster** on p.m.forster@leeds.ac.uk or **0113 343 6476**.

The salary, which is negotiable, will be within the Professorial range.

For more information and to apply, please go to www.universityofleedschairs.co.uk

Closing date: 8 June 2012.



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Peggy and Charles STEPHENSON CANCER CENTER — at The University of Oklahoma

FACULTY POSITIONS

The Peggy and Charles Stephenson Cancer Center at the University of Oklahoma Health Sciences Center invites applications for faculty positions. Rank and tenure eligibility to be commensurate with qualifications and experience. Interested applicants should have a demonstrated record of sustained, peer-reviewed funding and publications, with preference given to active NCI funding.

The SCC is Oklahoma's only academic cancer center, with over 80 members from the University of Oklahoma (OUHSC, OU Norman, OU Tulsa), the Oklahoma Medical Research Foundation, and Oklahoma State University. The SCC places a high priority on promoting translational research that moves research ideas into clinical applications. Position applicants should have demonstrated research in one of the following five SCC research programs (and research foci):

- Basic Cancer Biology (epigenetics/chromatin regulation; tumor microenvironment)
- Experimental Therapeutics (gene and drug delivery; nanomedicine; high-throughput drug discovery; cancer imaging; Phase 0 and Phase I trials)
- G.I. Cancers (cancer stem cell biology; epithelial mesenchymal transition; tumor/stromal interactions; inflammation and cancer; cell signaling/targeted therapy)
- Gynecologic Cancers (angiogenesis; drug resistance)
- Cancer Health Disparities (special populations, with emphasis in American Indian; health outcomes; tobacco research)

With the support of a recently completed \$50 million fundraising campaign and institutional development grants of over \$30 million (cancer research) and \$5 million (tobacco prev. and control research) from the Oklahoma Tobacco Settlement Endowment Trust, the SCC has launched an initiative to recruit 20 cancer-focused researchers over the next five years. Major investments are being made in developing the following SCC core facilities: Biospecimen, Bioluminescence Imaging, Cancer Genomics, Biostatistics, Special Populations, and Research Development/Proposal Services. The SCC supports a large clinical research program (annual enrollment of ~ 500 patients on therapeutic trials) and a rapidly expanding Phase I Program (anticipated 2012 enrollment of over 150 patients).

Applicants must possess an MD, an MD/PhD or a PhD in a relevant discipline and have a demonstrated potential for excellence in research. Selected candidates will have an appointment in an academic department as well as the SCC. For additional information, please visit www.stephensoncancercenter.org.

Applicants should provide electronic copies (only) of a cover letter stating area of expertise and qualifications, synopsis of professional goals, research interests, *curriculum vitae*, and e-mail addresses for three references to CancerResearch@ouhsc.edu.

Confidential enquiries may be addressed to Danny N. Dhanasekaran, PhD, Deputy Director for Basic Research, at danny-dhanasekaran@ouhsc.edu.

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Institutes of Biomedical Sciences of Shanghai Medical School, Fudan University

复旦大学上海医学院生物医学研究院

The Institutes of Biomedical Sciences (IBS) of Shanghai Medical School, Fudan University, is a scientific platform established in March 2005 and founded by the China National 985 Program. Aiming to foster and facilitate translational research, IBS has established a mechanism to link basic with clinical sciences. All investigators in IBS have access to shared state-of-the-art core facilities and equipment. During the last 7 years, 25 principal investigators have joined in IBS and their research has achieved publications in prestigious journals, including Science and Nature.

Principal Investigators Positions

Applicants should have a strong research profile to establish and conduct innovative research in life sciences. Both senior and junior investigators are encouraged to apply in the areas of cancer, epigenetics, stem cell, proteomics, structure biology, metabolic diseases, immunologist working on infection and vaccine.

As an eligible candidate for professorship, you should have:

- PhD degree and hold a position at assistant professor level or above, or equivalent position.
- High-profiled publications as corresponding author.
- < 40-years old is preferred

As a candidate for junior faculty position, you should have:

- PhD degree with postdoctoral experience.
- Good publication record.
- < 35 years old is preferred

The medical school and the university provide research facilities and strong supporting staffs. Internationally competitive start-up support, salary and benefits will be offered according to qualifications and experience.

Applications for faculty positions are open.

Contact person: **Junhui Bie**
Email: biejunhui@fudan.edu.cn
Tel: +86-21-54237847

THE UNIVERSITY OF SOUTH DAKOTA

Assistant or Associate Professor Sanford School of Medicine

The Division of Basic Biomedical Sciences at the University of South Dakota's Sanford School of Medicine invites applications for a tenure-track faculty position at the Assistant or Associate Professor level. Applicants should have a Ph.D. or M.D./Ph.D. in Microbiology, Immunology or Infectious Diseases or other related fields and at least two years of post-doctoral experience. We seek energetic, interactive individuals whose areas of interest include, but are not limited to, the study of mechanisms used by pathogens to exploit cellular processes and pathogen modulation of host cell responses. Preference will be given to candidates whose research complements existing strengths in the Division. The Division unites the classic basic science medical departments into a single administrative unit; a structure that breaks down traditional boundaries and allows interdisciplinary collaboration to flourish. In addition to infectious diseases other areas of research strength include cardiovascular diseases, oncology, cell and molecular regulation, neuroscience and protein quality control. (see <http://www.usd.edu/medical-school/biomedical-sciences/faculty-and-staff.cfm> for a complete list of faculty within the Division of Basic Biomedical Sciences). Successful candidates will be expected to develop an independent, externally funded research program and to participate in teaching graduate or medical students. Excellent start-up funds, state-funded salary commensurate with experience and state of the art research facilities in the new Lee Medical Science Building (Vermillion, SD) will be provided. Application should include curriculum vitae, a summary of past research and teaching experience, a statement of research interests and future plans, as well as the names of three references. All materials should be sent to The University of South Dakota online employment website at <https://yourfuture.sdbor.edu>. Review of applications will begin on June 15, 2012 and continue until position is filled. Women and minorities are encouraged to apply. AA/EOE.



TENURE TRACK FACULTY POSITIONS Department of Oral Biology

The Department of Oral Biology invites applications for multiple full-time, tenure-track faculty positions at the Professor, Associate Professor, and Assistant Professor level. We are seeking outstanding individuals capable of establishing and maintaining an independent research program. Research areas of interest include:

- Host-pathogen interactions pertaining to oral infections, and effects of the resulting innate/adaptive immunity on local and systemic health, including bone (osteoinmunology)
- Salivary research; mucosal immunity of the digestive and respiratory tracts
- Oral microbiome and microbiology in oral and overall health and disease

Candidates who bring expertise in immunological, biochemical, bioinformatics, and/or molecular biological approaches are encouraged to apply, as are those with dual degrees (e.g., DDS/PhD, DMD/PhD, MD/PhD).

The successful candidate will be expected to contribute to the teaching mission of the department, including supervision of graduate students in Oral Biology, and instruction in the undergraduate and graduate Dental School curricula. Candidates should hold DDS, DMD, MD, PhD, or equivalent. Successful candidates are expected to have or obtain significant research funding, a national/international research reputation, and appropriate teaching experience.

Formal applications for these positions must be made through the UBJobs website at: <https://www.ubjobs.buffalo.edu/applicants/Central?quickFind=55020>

The University at Buffalo is committed to increasing diversity within its faculty by seeking women and minority candidates.



KEEP AMERICA SAFE THROUGH SCIENCE AND TECHNOLOGY! Director, Chemical/Biological Defense Division

The Department of Homeland Security seeks an Executive who influences and develops policy regarding chemical or biological programs, and countermeasures that protect against biological and chemical attacks for the position of Director, Chemical/Biological Defense Division (CBD). This is a Senior Executive Service (SES) position, located in the Science and Technology Directorate (S&T), in the Homeland Security Advanced Research Projects Agency (HSARPA).

The CBD Director administers a \$100M Research & Development (R&D) portfolio to provide novel solutions aimed towards protecting the country from a Weapons of Mass Destruction attack. The Director is responsible for the management and leadership of the Chemical and Biological program, stewardship of assigned resources, and execution of the Division's programs including the cost, schedule, technical performance and deliverables associated with those programs.

The successful candidate will creatively leverage resources and partnerships to develop the next-generation architecture for chemical and biological defense, including bio-surveillance, point-of-care diagnostics, and real-time detection. In close cooperation with the White House and Executive branch agencies, the Director actively shapes policies and plans for detection, response and mitigation from a chemical or biological attack. You will find additional qualifications and responsibilities at www.usajobs.gov. The vacancy announcement number is **CHCO-12-016-DHS-645716**. Submit your application by **May 21, 2012**.

Technical Program Managers needed in all Disciplines!

HSARPA is also seeking Program Managers and subject matter experts in the following areas of expertise: Cyber Security, Borders and Maritime Security, Explosives, Human Factors and Infrastructure Protection & Disaster Management. If interested in a career opportunity in one of these areas of expertise, please send your resume/qualifications to STExecutiveResource@dhs.gov.

For more information about S&T can be found here: <http://www.dhs.gov/scienceandtechnology>.



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You will be a future research leader and we expect to work with you to ensure that you enjoy the support you require to develop as a world-leading scholar. You will join a vibrant interdisciplinary research community in the IAS and will also benefit from advanced training delivered through the IAS Academic Careers and Employment programme (ACE).

You will be expected to engage in PhD supervision and to improve your teaching skills as part of professional development. You will not be expected to undertake academic administration during your Fellowship term.

Excellent candidates from all fields are invited to apply. Applications from candidates with existing funding are also welcome. We welcome applications from candidates in the UK and overseas.

You will be based in the IAS but will have a strong presence in a home academic department. Therefore, all applications **MUST** be supported by a Warwick academic nominator and the proposed research project should complement the nominated academic department's research interests. Nominators will be required to make a strong case for the applicant's strategic contribution to the research within their department.

You should hold a doctorate or equivalent medical qualification and be within 5 years of your PhD graduation. You must have passed your viva and submitted the final copy of your thesis to be eligible. You should not have more than 5 years' postdoctoral research/teaching experience or hold, nor have held, a permanent academic post.

The posts are available from the beginning of October 2012.

For further details and an application form please visit our website below. More information about the IAS is available from www.go.warwick.ac.uk/IAS Minicom users: 024 7615 0554

The nomination form can be downloaded from the Warwick website (www.warwick.ac.uk/jobs). Applications without a signed nomination form will not be considered.

Interview date: End of June / beginning of July

Closing date: Monday 28 May 2012

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Systems Biology Faculty Position

The Peggy and Charles Stephenson Cancer Center at the University of Oklahoma Health Sciences Center is seeking applicants for a faculty position in Systems Biology. Rank and tenure eligibility will be commensurate with qualifications and experience. Stephenson Cancer Center members work in all aspects of cancer research, and the Center is promoting collaborative activities with an integrated systems biology approach among researchers within the Center and its affiliated institutions.

We seek exceptional candidates to lead a Systems Biology research focus at the Stephenson Cancer Center. Candidates should have demonstrated experience in combining experimental and computational approaches to elucidate the function, organization, and functional dynamics of the cancer genome. Candidates should be highly-motivated, have exceptional analytical skills, a strong interest in developing predictive cancer phenotypic models (predictive and/or prognostic biomarkers), and the ability and desire to work in a multidisciplinary team of cancer biologists, molecular biologists, clinical researchers, and oncologists. The selected candidate will be responsible for the integrative analysis of high-dimensional genetic and epigenetic data sets related to cancer genesis, progression, metastasis, and drug resistance with a focus on developing predictive diagnostic, therapeutic, and prognostic models and extending these experimental cancer models to clinical applications.

Applicants should have a Ph.D. or equivalent degree in a relevant area of cancer research including biochemistry, molecular biology, genetics, genomics, biomedicine, biophysics, bioengineering, chemistry, mathematics, computer science or statistics. Applicants should have relevant postdoctoral research training, demonstrated experience in interpreting cancer-relevant systems biology data sets, and a record of excellence in research as demonstrated through publications and funding. For additional information about the Cancer Center, please visit www.StephensonCancerCenter.org.

Applicants should electronically submit a *curriculum vitae*, a synopsis of professional goals and research interests, and arrange for at least three letters of recommendation to CancerResearch@ouhsc.edu. Confidential inquiries should be addressed to Danny Dhanasekaran, Ph.D., Deputy Director for Basic Research, Stephenson Cancer Center, at danny-dhanasekaran@ouhsc.edu.

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From the journal *Science*



**Georgia Health
Sciences University**

**Endowed Chair in Translational Neuroscience
Req. #6103**

The Medical College of Georgia (MCG) at Georgia Health Sciences University (GHSU) invites applications for a tenure-track full professor engaged in clinical research with a Neurologic/Immunologic/Inflammatory Diseases focus. This position comes with a substantial endowment and start-up package appropriate for an exceptional individual with a sustained record of productivity in a relevant research area, an international reputation and major national presence by virtue of positions on editorial boards, NIH study sections, and other leadership positions.

The successful candidate would be expected to establish and develop research programs that complement existing basic and clinical science groups at GHSU. Although the primary effort would be to conduct research, the successful applicant for this position will also be expected to participate in the typical range of academic activities such as clinical and/or educational service.

- M.D. or M.D., Ph.D. or equivalent is required.
- Ph.D. candidates with a sustained record of productivity in the research fields cited above, and an active commitment to supporting related clinical research are also encouraged to apply for this position.
- Salary and fringe benefits package are highly competitive and salary is commensurate with experience and qualifications.

GHSU has a long tradition of excellence in education and patient care and is one of four research-intensive universities of the University System of Georgia. It is comprised of colleges of Allied Health Sciences, Dental Medicine, Graduate Studies, Medicine, and Nursing and is the only academic institution in the state dedicated exclusively to the health sciences. MCG is the founding school of GHSU and is the state's only public medical college. Augusta, home of the Master's golf tournament and a charming Southern city, is a superb place to live with a low cost of living and a short drive to both the Georgia/Carolina mountains and the Georgia/Carolina/Florida coast. Salary and fringe benefits package are highly competitive.

For more information, please contact:

David Hess, M.D.

Professor and Chair

Department of Neurology

E-mail: dhess@georgiahealth.edu

Andrew Mellor, Ph.D.

Professor of Medicine and GRA Eminent Scholar

Director, Immunotherapy Center

E-mail: amellor@georgiahealth.edu

To apply online, please visit: www.georgiahealth.edu/facultyjobs

GHSU is an Equal Employment, Equal Access and Equal Educational Opportunity and Affirmative Action Institution. It is the policy of the university to recruit, hire, train, promote and educate persons without regard to age, disability, gender, national origin, race, religion, sexual orientation or veteran status.



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From technology specialists to patent attorneys to policy advisers, learn more about the types of careers that scientists can pursue and the skills needed in order to succeed in nonresearch careers.



POSITIONS OPEN

The College of Veterinary Medicine of Mississippi State University is seeking applications for a tenure-track **ASSISTANT/ASSOCIATE PROFESSOR** position in the Department of Basic Sciences. The successful applicant must have research experience and interest in an area that is relevant to human health disparities. Examples include type 2 diabetes, cardiovascular disease, neurological and/or behavioral disorders, and infectious diseases. Health disparities research has been identified as a university-wide research priority. The Center for Environmental Health Sciences in the College of Veterinary Medicine is active in health disparities research, and the role of exposure to environmental chemicals (pesticides, for example) in the occurrence of health disparities is a unifying theme within the Center. Individuals with a background in basic, mechanistic research who are willing to collaborate and initiate projects that will address toxicological aspects of health disparities are encouraged to apply as well as those with formal training in toxicology. The successful applicant will be expected to develop an active research program with extramural funding and to collaborate with other faculty in health disparities research. At the Associate Professor level, candidates with active, transferable research grants will be given preference. The successful applicant will be expected to participate in our instructional programs for graduate (M.S. and Ph.D.) and/or D.V.M. students. Minimum qualifications include a Ph.D., D.V.M., M.D., or other equivalent degree with postdoctoral research experience. Salary and rank will be commensurate with experience. Evaluation of applications will begin in June and continue until the position is filled. Please send a curriculum vitae, names and contact information for three persons who can provide letters of reference, and a brief (one page or less) statement of research plans and goals as well as teaching philosophy to: **Dr. Stephen B. Pruett, Department of Basic Science, College of Veterinary Medicine, P.O. Box 6100, Mississippi State, MS 39762.** E-mail communication is preferred (e-mail: pruett@cvm.msstate.edu). *MSU is an Affirmative Action/Equal Opportunity Employer, and members of minority groups who are underrepresented in the sciences are particularly encouraged to apply.*

ASSISTANT RESEARCH SCIENTIST

The University of Iowa, Department of Anesthesia is seeking an Assistant Research Scientist to join an active NIH-funded laboratory group that is part of the larger University of Iowa Pain Research Program. Research focuses on pharmacological, behavioral and electrophysiological methods to investigate how post-operative pain controls peripheral sensitization. Required qualifications include; a Ph.D. in the biomedical sciences or equivalent professional degree (e.g. M.D., D.D.S., or D.V.M.) as well as practical experience and theoretical knowledge of the field of neuroscience or pain, a minimum of three years postdoctoral experience including experience with electrophysiology, evidence of progressively responsible independent research work including publications in peer-reviewed journals, excellent organizational and communication skills, and ability to work independently and must be self-motivated with strong work ethic. For more information about the position and to apply online, go to **website: <http://jobs.uiowa.edu>** and reference **requisition #60821**. Applications will be considered until the positions are filled.

The University of Iowa is an Equal Opportunity/Affirmative Action Employer. Women and minorities are encouraged to apply.

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